

STUDIES ON BOVINE PETECHIAL FEVER
AND OVINE TICK-BORNE FEVER

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1974.



This thesis has been composed by
me, and describes my own work.

ACKNOWLEDGEMENTS

I wish to thank Dr. G.R. Scott for his great interest, advice, and helpful criticism throughout my work, and Mr. J.G. Tremlett for his help and encouragement during my stay in Kenya.

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SUMMARY

A comparative study was made of tick-borne fever in sheep in Scotland, and bovine petechial fever in sheep and cattle in Kenya. The results obtained demonstrated the similarity of the two diseases.

Both are caused by rickettsia-like organisms observable principally in the neutrophils, and tentatively classified in the genus Cytoecetes. These were considered to be morphologically identical.

Clinical signs and necropsy lesions of experimental animals were noted. The parameters of normal infections with both diseases were defined, with reference to haematological, febrile, and parasitaemic responses. The distribution of the causative organisms in infected hosts at different stages of reaction was investigated by infectivity studies; spleen and lung were found to contain the highest titres.

A latent infection was a more common sequel to tick-borne fever than to bovine petechial fever.

A complement fixation test was developed for tick-borne fever, antigen being prepared from infected leucocytes. Similar methods proved only partially successful for bovine petechial fever.

A hitherto unsuspected breed difference in susceptibility to bovine petechial fever was observed, and thereafter Sahiwal cross cattle were used routinely.

Investigations were made into the potential role of wild ruminants in the epidemiology of bovine petechial fever, by experimental inoculation of captive wild animals. Several species were found to allow multiplication; as a corollary to this, isolates of the causative organism were made from wild bushbuck in an enzootic area.

Three antibiotics were compared in the treatment of patent bovine petechial fever reactions in sheep, and the most efficacious of these (dithiosemicarbazone) was used successfully to treat the disease in cattle.

The similarities between the two diseases were so marked that it was concluded that the diseases may well be caused by different strains of the one microorganism.

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Background

Cases of a haemorrhagic syndrome in cattle on Ondiri farm, near Kikuyu, Kenya, were described by Danks (Kenya, 1933). This condition became known as bovine petechial fever (BPF), or more commonly in Kenya as ondiri disease or ondiriitis. Subsequent work in the 1930's was concerned mainly with clinical and postmortem descriptions, transmission of the disease by blood inoculation, and attempted transmission by ticks and biting flies (Kenya, 1935; 1936; 1937).

An increasing incidence and distribution of the disease occurring in the 1950's led to further work being carried out, culminating in the establishment of the aetiology by Hain and Tanskin (1962). Detailed light and electron microscope studies of the causal organism were undertaken by Krauss, Davies, Odegaard, and Cooper (1972). Reviews have been published by Piercy (1953), Tanskin and Lurdin (1963), and Hain (1966).

The causative agent described by Hain and Tanskin (1962) was a small rickettsia-like organism, observed principally in the neutrophils, but also in eosinophils and monocytes. The name Cytoecetes ondiri was proposed by Krauss et al (1972). Another probable member of this genus is C. phagocytophila (Fozzard, 1962a), the causal agent of tick-borne fever (TBF).

The purpose of the study was to obtain more information on the pathogenesis and epidemiology of BPF, as very little was known about these aspects. However, considerable work has been carried out on TBF, in sheep principally by Dr. A. Foggie of the Moredun Institute, and in cattle by Dr. J. Tuomi of Helsinki. It was therefore decided to use TBF in sheep in Scotland as a model to develop methods for the study of BPF in cattle in Kenya.

To this end, 18 months were spent at the Centre for Tropical Veterinary Medicine, University of Edinburgh, Scotland, and 2 years at the Veterinary Research Laboratories, Kabete, Kenya.

Classification

The study of these 'rickettsia-like' organisms has to be considered within the framework of their taxonomic position. Members of the genus Rickettsia are small gram-negative, intracellular coccobacilli; they are pathogenic to man, but are adapted to and transmitted by arthropods. 'Rickettsia-like' applied to those organisms pathogenic to animals implies a similar morphology and a probable arthropod transmission. Animals may act as reservoir hosts for Rickettsia pathogenic to man e.g. R.tsutsugamushi in rodents, and R.conorii in dogs. However, this thesis is concerned with rickettsia-like organisms pathogenic to animals.

The first such organism was described from heartwater by Cowdry (1925) in the endothelial cells of infected animals. This organism is now accepted as Cowdria ruminantium, but no other members of the genus Cowdria have been described (Philip, 1957).

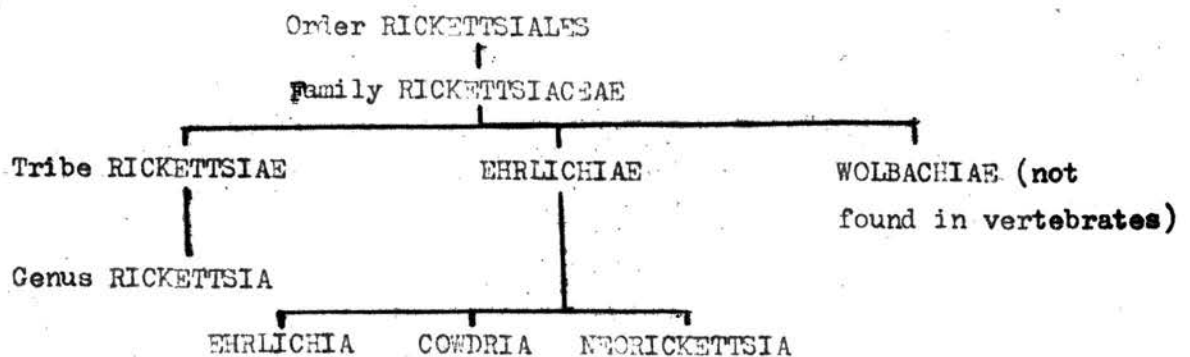
A group of rickettsia-like organisms parasitising monocytes was described by French workers in North Africa: R.canis (Donatien and Lestoquard, 1935); R.bovis (Donatien and Lestoquard, 1936); R.ovina (Lestoquard and Donatien, 1936); and possibly R.suis (Donatien and Gayot, 1943). These are now accepted in the genus Ehrlichia (Moshkovskii, 1945), thus E.canis, E.bovis, and E.ovina (Philip, 1957), but no mention is made of R.suis.

E.canis is the only one of these organisms to have been studied subsequently, because of its occurrence in the U.S.A. (Ewing, 1963),

and in British and American war-dogs in Southeast Asia (Wilkins, Bowden, and Wilkinson, 1967; Huxsoll, Hildebrandt, Nims, Ferguson, and Walker, 1969).

Another rickettsia-like organism causes 'salmon poisoning' in dogs on the Pacific coast of the U.S.A., and occurs in cells of the reticuloendothelial system. It is in a separate genus as Neorickettsia helminthoeca, and has no arthropod vector.

Thus the classification proposed by Philip (1957) is:



Unfortunately, the otherwise comprehensive classification proposed by Philip (1957) makes no reference to either the genus Cytoecetes, or to the existence of these intraneutrophil organisms. The first reference to this genus was made by Tyzzer (1938), who described the type species C.microti in voles from Massachusetts. Since then, in addition to C.ondiri and C.phagocytophila, two other organisms have been described which seem from their descriptions to be Cytoecetes, but for which no generic position has been proposed: that causing equine ehrlichiosis (Stannard, Gribble, and Smith, 1969), and that causing an atypical canine ehrlichiosis (Ewing, Roberson, Bickner, and Hayat, 1971). It would seem logical that the genus Cytoecetes should be placed alongside the genus Ehrlichia in the tribe Ehrlichiae, as suggested by Krauss et al (1972).

During this introduction, therefore, the other organisms that will be considered as having most relevance to BPF and TBF are Cytoecetes and Ehrlichia spp, and Cowdria ruminantium.

Occurrence

BPF has been recognised only from Kenya, at altitudes over 5000 feet. It occurs mainly in pure-bred or high-grade stock (Piercy, 1953), and is nearly always associated with grazing at forest edge or thick scrub (Danskin and Burdin, 1963), often on a restricted part of a farm, or even part of one paddock. Walker, Cooper, and Snodgrass (1974), in an epidemiological study, found that the only characteristic common to all sites was thick bush or understorey cover giving heavy shade, with high relative humidity in a thick litter layer.

Although seasonal occurrence after rain has been noted (Kenya, 1937; Plowright, 1962), others have found little seasonal variation in incidence (Piercy, 1953; Walker et al, 1974).

TBF occurs seasonally in sheep and cattle in northern Europe, and this is associated with the feeding habits of the vector tick Ixodes ricinus (MacLeod and Gordon, 1933; Tuomi, 1966).

Clinical Signs

The incubation period following natural BPF infection is not known, but cases have been observed within 10 days of movement of animals into infected areas. Following artificial infection the incubation period was in the range 5-14 days (Haig, 1966).

Typically, the first sign was a rise in temperature to about 41°C, followed by the appearance of numerous haemorrhages under the tongue and on other external mucosae. Often other signs were limited, making the drop in milk yield observed in dairy cows more obvious

(Kenya, 1933; 1935; 1936).

More detailed accounts of the clinical symptoms were given by Piercy (1953), Danskin and Burdin (1963), and Haig (1966). Petechiae were observed one to two days after the initial temperature rise. These were particularly noticeable on the lower surface of the tongue and on the vulva, but were also seen on the lips, gums, nose, conjunctiva, and rectum. Plowright (1962) stressed the characteristic appearance of the petechiae on a congested background.

Superficial lymph nodes were enlarged, and on occasion a watery ocular discharge and epistaxis were observed. A characteristic symptom sometimes seen was 'poached egg eye', consisting of swelling and protrusion of the conjunctiva, with blood loss into the anterior chamber.

Anorexia was not common. In acute cases faeces were tarry, or occasionally contained whole blood.

Pregnant animals usually aborted.

The severity varied from inapparent to fatal, about 20% of the animals dying in the acute phase with respiratory distress. Others made a slow recovery.

In sheep artificially infected with BPF, signs were limited to high fever (Dawe, Ohder, Wegener, and Bruce, 1970).

The clinical signs of TBF in sheep were first described by Gordon, Brownlee, Wilson, and MacLeod (1932) and MacLeod and Gordon (1933). Following an incubation period of about 4 days after inoculation, the temperature rose sharply, and remained irregularly high for about 10 days. During the febrile reaction there was dullness, and often loss of weight. Most sheep made an uneventful recovery.

Since then, however, there are reports of more severe illness in

sheep associated with TBF or its side-effects. The most dramatic of these was the report of 25% mortality in young sheep (Jamieson, 1947). This is so at variance with other results that, although TBF was present, there must be some doubt as to whether it alone was responsible. Abortion is often associated with natural and experimental TBF in sheep (Stamp and Watt, 1950), but whether this is a specific effect of TBF infection, or due to the febrile reaction, is not known. The disease has also been reported to cause transient infertility in rams (Watson, 1964), and a haemorrhagic enteritis (Foster, Foggie, and Nisbet, 1968).

However, it is the aggravation of other disease conditions by TBF that is most important. It has been suggested that louping-ill virus will only invade the sheep's central nervous system in the presence of concurrent TBF infection (MacLeod, 1962b); TBF is associated with staphylococcal pyaemia of lambs (Foggie, 1956; Foster and Cameron, 1968a), which is attributed to the neutropoenia of TBF infection; and there seems to be a predisposition to secondary pneumonias (Foggie, 1951), perhaps for the same reason.

TBF in cattle appears to be milder than the high fever would indicate (Hudson, 1950). In milking cows, the most marked sign is fall in milk yield, while in other cattle slight depression and coughing have been noted (Hudson, 1950; Tuomi, 1967a).

A severe outbreak of abortion has been described (Wilson, Foggie, and Carmichael, 1964) and occasional deaths with a haemorrhagic syndrome (Foggie and Allison, 1960; Wilson et al, 1964).

Equine ehrlichiosis is also a mainly mild disease, characterised by multiphasic febrile reaction, depression, and oedema of the limbs (Gribble, 1969). Atypical canine ehrlichiosis due to Cytoecetes is a mild febrile condition (Hayat, Ewing, and Buckner, 1972).

Canine ehrlichiosis due to E.canis is a mild febrile reaction in mongrels, but more severe in Beagles, and leads to tropical canine pancytopenia (TCP) in Alsations (Seamer and Snape, 1972; Huxsoll, Amyx, Hemelt, Hildebrandt, Nims, and Gochenour, 1972). Dogs with TCP show marked haematological changes, and some show severe epistaxis, usually accompanied by oedema, depression, mucosal petechiae, and corneal opacity (Walker, Rundquist, Taylor, Wilson, Andrews, Barok, Hogge, Huxsoll, Hildebrandt, and Nims, 1970).

E.bovis infection and E.ovina infection were both mild diseases when described by Donatien and Lestoquard (1937), but have since been found to produce severe illness with high mortality in exotic livestock in Senegal (Rioche, 1966).

Haematology

Marked haematological changes are a feature of both BPF and TBF. In BPF there occurs a leucopenia, thrombocytopenia, and anaemia (Kenya, 1953; Danskin and Burdin, 1963). Krauss et al (1972) noted lymphopenia followed by neutropenia, while Flouwright (1962) found mainly a decrease in mononuclear cells and a complete absence of eosinophils.

Similar changes occur in TBF, with lymphopenia followed by neutropenia described in sheep (Taylor, Holman, and Gordon, 1941) and cattle (Tuomi, 1967a), and thrombocytopenia by Foster and Cameron (1968b).

The haematological changes in equine ehrlichiosis are also similar to these (Gribble, 1969). Much more detailed studies have been made on natural tropical canine pancytopenia, a severe manifestation of E.canis infection (Walker et al, 1970), and on experimental uncomplicated canine ehrlichiosis (Seamer and Snape, 1972). They report

decreases in leucocytes, erythrocytes, packed cell volume, and haemoglobin, with an increased erythrocyte sedimentation rate and blood urea nitrogen. Mean corpuscular haemoglobin concentration and mean cell volume did not vary significantly.

In E.bovis infection, there is an increase in monocytes, and eosinophils usually disappear (Rioche, 1967).

Latency

The possible development of latent infection after recovery from BPF has been little studied. Danskin and Burdin (1963) found that the blood of cattle was still infective 4 days after clinical reaction.

Foggie (1951), by subinoculation of 1 ml of blood from recovered sheep, showed that C.phagocytophila may persist for more than 2 years after TBF. With TBF in cattle, Tuomi (1967a) failed to demonstrate latency by 1 ml subinoculations, but succeeded with a 500 ml subinoculation taken 9 days after the initial reaction.

No data are available on latency in the other Cytoecetes spp., but latency is a characteristic of the related organisms: after E.canis infection for up to 29 months (Ewing and Buckner, 1965); after E.ovina infection (Lestoquard and Donatien, 1936); after E.bovis infection (Parrot, 1937); and after infection with Rickettsia spp. (Fox, 1948). C.ruminantium may persist in some recovered animals (Donatien and Lestoquard, 1937), but Haig (1955) was not able to confirm this.

Immunity

Immunity to BPF after natural and experimental infection in cattle is known to last for at least 1½ to 2 years, and to be effective between different isolates (Kenya, 1936; Piercy, 1953; Kenya, 1958; Danskin

and Burdin, 1963). The only reported work on immunity in experimental sheep found homologous strain immunity declining from 3-4 weeks after infection (Dawe et al, 1970).

Papers written on TBF present a confusing picture regarding immunity. After natural or experimental infection, some workers have found sheep and cattle susceptible within 3-12 months (Overas, 1962; Gordon et al, 1932; Jamieson, 1947; Hudson, 1950; Foggie, 1951). Others have found good immunity to natural and experimental challenge, or reported the occurrence of TBF only in bought-in animals, which implies good immunity (Venn and Woodford, 1956; Wilson et al, 1964; Stamp and Watt, 1950; Tuomi, 1967a). Tuomi (1967c) found poor heterologous strain immunity, but any interpretations of heterologous challenges must bear in mind the uncertainty regarding homologous immunity.

Equine ehrlichiosis confers a strong immunity for up to 20 months, the longest period tested (Gribble, 1969). Animals recovered from infection with E.canis, E.bovis, and E.ovina develop good immunity to challenge (Donatien and Lestoquard, 1937), but immunity after heart-water infection is variable (Haig, 1955).

Morphology of causal organisms

C.ondiri as the cause of BPF was first described by Haig and Danskin (1962), but a fuller description of the light and electron microscopic appearance has been given since by Krauss et al (1972). The latter described small bodies of about 0.4 μ , larger bodies of 1-2 μ , groups of small and large bodies, and groups of small bodies which were termed morulae, all staining blue with Giemsa. The morulae predominated later in the infection cycle. The organisms were in a

cytoplasmic vacuole with a limiting membrane. Binary fission was observed, as was the breakdown of giant bodies to small bodies.

Other related organisms are generally similar. All are present in cytoplasmic vacuoles with a limiting membrane; binary fission has been observed in all; and in C.ruminantium a process of breakdown of giant bodies has been noted.

C.phagocytophila was described under light (Hudson, 1950; Foggie, 1951) and electron (Tuomi and von Bonsdorff, 1966) microscopy. Its description was almost identical to that of C.ondiri, as was that of the organism causing equine ehrlichiosis (Gribble, 1969).

C.ruminantium was described by Haig (1955) under light microscopy and Pienaar (1970) under electron microscopy. There is a small discrepancy between the particle sizes, given by the former author as $0.3 - 1.7 \mu$, and by the latter as $0.5 - 2.7 \mu$.

E.canis particles were described by Haig (1955), varying in size from $0.2 - 1.5 \mu$. Simpson (1972) observed E.canis under electron microscopy, and found a particle size of 0.75μ . However, this was a very limited study, and other particle sizes may exist.

Serology

Unsuccessful attempts have been made to demonstrate specific antibody in BPF by neutralisation in vitro, gel precipitation, and prophylaxis or therapy with convalescent and hyperimmune sera (Kenya, 1956; 1957).

No crossreaction has been found with chlamydial antigens and sera from cases of BPF (Krauss et al, 1972), TBF (Foggie, 1962b), canine ehrlichiosis (Nims, Ferguson, Walker, Hildebrandt, Huxsoll, Reardon, Varley, Kolaja, Watson, Shroyer, Elwell, and Vacura, 1971),

and heartwater (Rake, Alexander, and Hamre, 1945). There was no serological relationship between rickettsial antigens and sera from E.canis infection (Nims et al, 1971).

Experimental host range

C.ondiri has been successfully transmitted to goats and sheep, producing a reaction with fever and parasitaemia, but no haemorrhages (Danskin and Burdin, 1963). There are early reports of the susceptibility of dogs and rabbits (Kenya, 1935; 1936). These latter results have never been repeated, and as the identity of the aetiological agent was then unknown, such results must be suspect. Attempts to infect horses, guineapigs, rats, mice, and hamsters have failed (Cooper, 1973; Kenya, 1937; 1956; Danskin and Burdin, 1963; Dawe et al 1970).

C.phagocytophila has been adapted to splenectomised and normal guineapigs and splenectomised mice (Foggie and Hood, 1961), and C.ruminantium has been successfully transmitted to ferrets (Mason and Alexander, 1940) and mice (du Plessis and Kumm, 1971). Nevertheless, these experimental hosts have not been used in further studies.

Laboratory rodents were not susceptible to E.canis (Ewing, 1969). Mice were resistant to E.bovis, but this organism has been transmitted to sheep (Donatien and Lestoquard, 1937) and pigs (Rioche and Bourdin, 1968). The agent of equine ehrlichiosis has been transmitted to sheep, goats, and dogs (Gribble, 1969).

Experimental and natural infections of wild animals with these organisms is described in the section on epidemiology.

In vitro propagation

Attempts to propagate C.ondiri in embryonated eggs and in various cell culture systems failed (Danskin and Burdin, 1963; Dawe et al, 1970; Davies, F.G., personal communication). Similarly, C.phagocytophila has not been cultivated in vitro (Hudson, 1950; Foggie, 1951; Tuomi, 1967a; Thrushfield, M., personal communication).

Nyindo, Ristic, Huxsoll, and Smith (1971) successfully cultured infected leucocytes from E.canis-infected dogs, but no further use of this technique has been made. Attempts to propagate E.bovis in eggs and cell culture also failed (Rioche and Bourdin, 1968).

Therapy

A variety of antibiotics and other drugs have been tested for activity against BPF, both in vitro, during the incubation period, and in clinical disease (Kenya, 1956; 1957; 1959; 1960; Haig and Danskin, 1962).

There is a practice widespread in Kenya of treating BPF by intravenous inoculation of a turpentine/liquid paraffin mixture, for which great success is claimed by many farmers and veterinarians. Danskin and Burdin (1963) found it of no value in experimental cattle.

More detailed studies have been made of the therapies of TBPF (Tuomi, 1967e; Evans, 1972), E.canis infection (Buckner and Ewing, 1967; Walker et al, 1970), and heartwater (Neitz, 1939; Weiss, Haig, and Alexander, 1952; Haig, Alexander, and Weiss, 1954; Haig, 1955). The susceptibility of the different organisms was similar in all cases: chlortetracycline and oxytetracycline were highly efficacious, sulphonamides moderately so, and penicillin, streptomycin, and chloramphenicol were inactive. The most efficacious treatment for E.bovis infection was chlortetracycline (Rioche, 1966).

Dithiosemicarbazone has been found to be more effective than oxytetracycline in treating TBF (Evans, 1972), and has also been used to treat canine ehrlichiosis (Seamer and Snape, 1972).

Postmortem examination

The clinical picture of BPF as a haemorrhagic syndrome was confirmed on postmortem examination (Kenya, 1933; 1935; 1936). More complete reports have been given since by Piercy (1953), Kenya (1956), Danskin and Burdin (1963), Haig (1966), and particularly by Plowright (1962) in a quantitative study of 25 field cases.

In addition to haemorrhage, oedema and lymphoid hyperplasia were characteristic of the disease.

Subcutaneous and intramuscular haemorrhages and oedema were usually seen. The most consistent abnormalities were seen in the heart, where there were extensive subendocardial and subepicardial haemorrhages, and excess pericardial fluid. The respiratory system sometimes showed haemorrhages in the laryngeal, tracheal, and bronchial mucosae, but the lungs were severely affected with oedema and haemorrhage only in peracute cases, otherwise showing slight congestion and oedema.

The urinary bladder almost always showed mucosal petechiae, while oedema and haemorrhage were observed in the mucosa of the gall bladder.

Haemorrhages were present throughout the length of the alimentary canal. In the small intestine they were on the serosal surface, while in the large intestine they were on the mucosal surface, giving rise to free blood and tarry faeces in the lower bowel.

Lymph nodes were usually enlarged with cortical and subcapsular petechiae, oedema, and hyperplasia. The spleen and liver were sometimes enlarged and showed petechiae. The kidneys and adrenal glands

on occasion showed cortical petechiae, with exudation of blood into the perirenal fat. The brain tissue was not affected, although congestion of the meninges was noted infrequently.

An interesting observation by Danskin and Burdin (1963) was the presence of cardiac haemorrhages in fetuses of 2 infected dams.

Histologically, little of significance other than petechiation has been observed, with the exception of the hyperplasia of the larger cells of the lymphoid series noted by Plowright (1962).

No significant macroscopic lesions have been found in animals killed during TBF reaction, other than slight increase in spleen size noted in cattle by Hudson (1950) and in sheep by Gordon et al (1932). Hudson (1950) also refers to mild degenerative changes in liver and kidney, and a depletion of the lymphoid tissue in spleen and lymph nodes.

Gribble (1969) records postmortem findings from a group of horses killed during reaction to equine ehrlichiosis. Gross lesions were mainly haemorrhages and oedema, as in BPF, but confined mainly to the subcutaneous tissue of the limbs. A third of the horses had increased amounts of peritoneal or pericardial fluid. Jaundice and orchitis were often noted. On histological examination, proliferative and necrotising lesions of small arteries and veins were seen, with perivascular accumulations of mainly mononuclear cells.

These vascular lesions are similar to those caused by Rickettsia spp. (Snyder, 1965).

Hildebrandt, Huxsoll, Walker, Nims, Taylor, and Andrews (1973) examined tissues from a large number of natural cases of tropical canine pancytopenia. Gross lesions of oedema, haemorrhage, and lymphoid hyperplasia were described. They also noted a perivascular

accumulation of mononuclear cells in meninges and kidneys, a hyperplasia of lymph nodes and spleen, and a hypoplasia of bone marrow. Uncomplicated canine ehrlichiosis causes hyperplasia of the bone marrow (Ewing, 1969).

The similarities extend to lesions of heartwater, where oedema and haemorrhage are accompanied by hydropericardium and hydrothorax (Haig, 1955). Perivascular leucocyte accumulations have also been noted, but the presence of the organism in endothelial cells seems merely to lead to distension.

E.bovis infection also produces haemorrhage and oedema, but perivascular accumulations of cells were not observed (Rioche, 1967).

There is thus a considerable degree of similarity between the pathological changes associated with the diseases caused by this group of organisms.

Epidemiology

Because of the sporadic nature of BPF, and its geographical confinement, it has long been suspected that it is transmitted by an arthropod vector (Kenya, 1936). Many unsuccessful attempts have been made to find such a vector, involving both experimental feeding on laboratory-infected animals, and collection of arthropods from the field. Ticks of the genera Rhipicephalus, Amblyomma, and Boophilus, have been used, and biting insects of Stomoxys, Culex, Anopheles, and Simulium genera (Kenya, 1937; 1957). A successful transmission by inoculation of emulsified engorged B.decoloratus from a reacting animal was presumed due to presence of fresh blood.

Haig (1966) suggested that the forest ticks R.hurti and R.kochi might be potential vectors, as their distribution was similar to that of BPF.

An investigation into the possible role of trombiculid mites found only low numbers of mites on rodents and cattle on infected farms, and these proved noninfective after emulsification and inoculation (Walker et al, 1974).

TBF is transmitted by the tick Ixodes ricinus in Scotland (MacLeod and Gordon, 1933), Norway (Thorshaug, 1940), and Finland (Tuomi, 1966). MacLeod (1962a) suggested that other ticks may be involved in TBF transmission in south-west England.

The vector of equine ehrlichiosis is not known, but most other related organisms are known to be tick-borne: C.ruminantium by Amblyomma spp.; E.bovis by Hyalomma spp.; E.ovina by Rhipicephalus spp. (Neitz, 1956); and E.canis by R.sanguineus (Donatien and Lestoquard, 1937). Stage-to-stage transmission is the norm, although transovarian transmission can occur with E.canis (Donatien and Lestoquard, 1937). Rickettsia spp. are also arthropod-borne.

Some farms experience continuing sporadic losses from BPF, while others experience no clinical cases unless animals are bought-in. This is presumed due to an enzootic state on these latter farms, with initial calfhoo infection while protected by maternal immunity, and subsequent repeated infection to boost immunity. On farms where sporadic cases occur, the level of challenge is possibly not high enough to maintain all animals in an immune state. Other farms no longer have any cases of BPF: one of these is Ondiri Farm, where bush clearance is presumed to have made the habitat not suitable for the vector.

The existence of a reservoir host has also been suggested as an explanation for the sporadic occurrence of the disease (Piercy, 1953; Haig, 1966). Of domestic ruminants other than cattle, sheep and goats are readily susceptible (Danskin and Burdin, 1963), and there are

unconfirmed reports of natural infection in sheep at Naivasha (W.A. Ashford, personal communication). No attempts have been made to infect experimentally species of wild ruminants, but efforts have been made to isolate the organism from mongooses (various genera), genet cats (Genetta spp.), cane rats (Thryonomys swinderianus), porcupine (Hystrix galeata), waterbuck (Kobus ellipsiprymnus), and small rodents in enzootic areas (Kenya, 1958; UNDP, 1972; Walker et al, 1974).

C. phagocytophila has been isolated from red deer (Cervus elaphus), roe deer (Capreolus capreolus), fallow deer (Dama dama), and wild goats (Capra hircus), in addition to sheep and cattle (Foggie, 1962a; McDiarmid, 1965; Foster and Grieg, 1969). Tuomi (1966) found an elk calf (Alces alces) to be resistant to TBF.

Red foxes (Vulpes vulpes), gray foxes (Urocyon cinereoargenteus), and coyotes (Canis latrans), have been experimentally infected with E. canis (Amyx and Huxsoll, 1973; Ewing, Buckner, and Stringer, 1964), and jackals (Canis mesomelas) and wild dogs (Lycaon pictus) may act as reservoir hosts in South Africa (Neitz and Thomas, 1938). C. ruminantium has been found in natural infection in springbok (Antidorcas marsupialis) (Neitz, 1944), while blesbok (Damaliscus albifrons) and black wildebeest (Connochaetes gnu) have been experimentally infected (Neitz, 1938). Gribble (1969) speculated that the horse may be an aberrant host in equine ehrlichiosis, and that sheep or deer might act as reservoir hosts.

The work described in this thesis was concerned with several different aspects of TBF and BPF infections, and so does not readily lend itself to description in general sections on materials and methods, results, etc. For this reason, the format adopted is that of separate chapters each dealing with an individual aspect of the work.

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TBF

CHAPTER 1GENERAL MATERIALS AND METHODS

1. Animals. Cheviot, Cheviot cross, or Suffolk cross sheep of either sex from tickfree farms in the south of Scotland were used. They were found to be uniformly susceptible to TBF.

Animals were housed throughout the experimental period.

2. C.phagocytophila. One strain of C.phagocytophila was used throughout. It was isolated from Sourhope in the Scottish Borders by Dr. W.N.M. Foster.

3. Infection. Blood from a parasitaemic sheep was used as inoculum. Amounts used were usually 1.0 ml of 10^{-1} dilution in phosphate buffered saline (PBS, pH 7.2) from a low-temperature storage bank. Inoculations were by the intravenous route.

4. Observations. Initially, rectal temperatures and blood samples in dipotassium ethylenediamine tetra-acetic acid (EDTA) were taken daily from all sheep. It became apparent that visible parasitaemia was always associated with a temperature in excess of 40.5°C . Thereafter sheep were bled only when they showed such a febrile reaction, unless a quantitative result was desired.

Blood smears were made, stained with Giemsa stain, and examined under an oil immersion X100 lens for the presence in the granulocytes or monocytes of C.phagocytophila (Foggie, 1951).

The presence of an inclusion body with the typical structure of C.phagocytophila was taken as evidence of infection. Before recording a sample as negative, at least 100 neutrophils were examined.

To quantify the magnitude of parasitaemia, total leucocyte counts were performed by electronic counter, and differential leucocyte counts by standard techniques (Dacie and Lewis, 1966). The percentage of TBF infection in at least 100 neutrophils was estimated, and the magnitude was then expressed as the number of infected neutrophils per cu.mm. The total magnitude of an animal's reaction may be expressed in terms of the area bounded by the graph of daily reaction values, calculated by the trapezium rule. However, when initial and final values are zero, as in this case, the area becomes the total of the individual points multiplied by a constant factor which is arbitrarily determined by the size of the graph paper grid. I have therefore omitted the constant factor and expressed the total parasitaemia magnitude as the sum of the daily values.

5. Cryopreservation of *C.phagocytophila*. The method of Foggie, Lumsden, and McNeillage (1966) was used. Infective blood was collected in heparin at 20 u/ml, and dimethyl sulphoxide (DMSO) added to give a final concentration of 10%. The suspension was mixed, and distributed in 1 ml amounts to glass bottles. These were cooled slowly by spending 30 minutes at each of 4°C and -20°C, and then transferred to -76°C or to nitrogen vapour at -196°C.

Before use the blood was thawed quickly under running water, and kept on ice until inoculated. The whole process was completed in under 30 minutes.

Two batches of blood 1 passage level apart were sufficient for most of the inocula needed. One stored at -76°C dropped 1 log₁₀ in titre on storage, and thereafter over 10 months showed a gradual decrease in titre, as evidenced by a lengthening of the incubation period from 3-4 days to 5-6 days (chapter 2). Eventually only large doses were infective.

TBF

The blood stored at -196°C also dropped in titre on storage, but thereafter showed no detectable loss in titre for the 6 months that it was used.

Other materials and methods are described in the relevant chapters.

CHAPTER 2

CLINICAL PARAMETERS

Clinical signs in TBF infection are minimal. The only consistent changes associated with the infection are fever, haematological changes, and visible parasitaemia.

This chapter records detailed changes in leucocytes, fever, and parasitaemia in sheep infected with TBF.

Materials and Methods

1. Animals. Twenty Cheviot sheep were inoculated with infected sheep blood. Dilutions were made in PBS and sheep were inoculated with 1.0 ml of dilutions from 10^0 to 10^{-4} .

In a further experiment, 8 sheep were inoculated from the same batch of blood, with 1.0 ml of 10^{-1} dilution.

2. Observations. Quantitative observations were made, usually at 24-hour intervals. Observations on the group of 8 sheep were made at 6-hour intervals during the period of rising parasitaemia.

Results

1. Parasitaemia. The parasitaemias in the sheep receiving different doses were compared (Fig. 1). The prepatent period to onset of parasitaemia varied inversely with the dose. The regression of dose on prepatent period fitted the equation

$$Y = -3.69 + 1.41X \quad (r = 0.865, p < 0.01)$$

where X was the prepatent period in days, and Y was the negative \log_{10} of the dose dilution.

Peak parasitaemia, the day of reaction on which the peak occurred, and the duration of parasitaemia, were not significantly different ($p > 0.01$), between the different dose levels.

To eliminate variation of prepatency, the results for the different doses were therefore pooled, taking as the starting point the first day of parasitaemia (Fig. 2, Table 1).

Maximum parasitaemia of $10^{3.4 \pm 0.2}$ infected neutrophils per cu.mm. was observed on day 1.6 ± 0.6 of reaction. The duration of parasitaemia was 6.2 ± 2.2 days. Mean total parasitaemia magnitude was 17.2 ± 6.2 .

41% of parasitaemias were monophasic, the remainder having two or three peaks (Fig. 3).

The results for parasitaemia from sampling at 6-hour intervals are shown in Fig. 2 and Table 2. They correspond to the figures obtained from 24-hour sampling.

2. Fever. The onset of reaction was usually marked by a rise in temperature (Fig. 4, Table 3). The mean incubation period to fever greater than 40.5°C was 3.9 ± 0.8 days, in the range 3-5 days. The incubation period to fever was the same as the prepatent period to parasitaemia in 74% of sheep. In the others, one preceded the other by no more than one day.

The reactions had a peak of $41.5 \pm 0.4^{\circ}\text{C}$ occurring on day 1.7 ± 0.9 of reaction. Fevers remained over 40°C for 7.5 ± 3.3 days.

35% of febrile reactions were monophasic. The other 65% had up to 6 peaks (Fig. 5). The maximum recorded temperature in the second and subsequent peaks was not significantly lower than in the first peak ($t = 1.74$, $p > 0.05$). There was no correlation between the

phases of temperature and parasitaemia reactions, only 2 sheep having monophasic reactions in both.

3. Haematology. Total leucocyte numbers decreased during reaction, from a preinoculation level of $12,600 \pm 2,900$ per cu.mm. to a mean nadir of $6,100 \pm 1,100$ per cu.mm. on day 5.0 ± 2.6 of reaction (Fig. 6, Table 4). This fall was made up of falls in the individual white cell types.

Eosinophil levels showed the most marked falls, eosinophils being absent on at least one day from the 1st - 6th day of reaction, in all sheep except one (Fig. 6, Table 5).

Lymphocytes counts dropped from $6,900 \pm 1,700$ per cu.mm. before inoculation, to $2,600 \pm 900$ on day 2.4 ± 1.0 of reaction (Fig. 6, Table 6). Neutrophils fell from a preinoculation level of $3,800 \pm 1,200$ per cu.mm. to a mean nadir of $1,000 \pm 600$ per cu.mm. on day 9.1 ± 1.9 (Fig. 6, Table 7). The lymphocyte nadir was significantly earlier than that of the neutrophils ($t = 14.6$, $p < 0.01$).

Eosinophil and neutrophil levels remained depressed for at least 2 weeks, but lymphocyte counts returned to normal levels after one week of reaction.

Monocyte counts changed less consistently than the other leucocyte counts, showing a rise from 380 ± 330 to 620 ± 210 per cu.mm. on the 5th day of reaction ($t = 2.30$, $p < 0.05$) (Table 8).

Discussion

The shape of the parasitaemia growth curve for TBF conforms to that of a typical biological growth curve (Monod, 1949). The prepatent period varied inversely with the dose; there was a period of rapid

exponential growth, a peak, and then a more gradual decline.

Previous workers have presented parasitaemia results as a percentage of infected neutrophils (e.g. Tuomi, 1967a). This takes no account of the marked changes in neutrophil levels. A rise of 10% in percentage infection might be accompanied by a fall of 20% in neutrophil levels, which would cause a drop in total parasitaemia, but which would have been recorded as a rise. The number of infected neutrophils per cu.mm. is a more accurate parasitaemia estimation. The omission of parasitised monocytes and eosinophils from this produces only a slight underestimation of total parasitaemia.

The haematological changes were marked, and conformed to the described pattern of lympho^poenia followed by granulocytopenia (Taylor et al. 1941). Lymphocyte and neutrophil levels both decreased by more than 50%, while eosinophils were almost uniformly absent. The fall in eosinophils coincided with the fall in lymphocytes.

The pooled graphs for fever and parasitaemia masked the typical multiphasic appearance of these reactions. The graphs showing mono- and multi-phasic reactions present a more normal type of reaction.

The quantitative figures given provide a degree of definition of the 'normal' reaction, and may be useful for comparison of different strains of BPF, or reaction in different animals.

Table 1. No. of observations, mean (\bar{x}), and standard deviation (sd), of parasitaemias (\log_{10} infected neutrophils per cu.mm.) in sheep infected with TBF.

Day of reaction	No. positive no. examined	\bar{x}	sd.
1	20/20	2.96	0.59
2	19/19	3.22	0.20
3	18/18	3.08	0.20
4	16/16	2.84	0.29
5	12/15	2.22	1.19
6	10/14	1.92	1.32
7	8/14	1.48	1.37
8	5/14	0.86	1.24
9	4/14	0.62	1.05
10	2/14	0.29	0.74

Table 2. No. of observations, mean (\bar{x}), and standard deviation (sd), of 6-hourly parasitaemia counts (\log_{10} infected neutrophils per cu.mm.) in sheep infected with TBF.

Time of reaction (6-hour intervals)	no. positive no. examined	\bar{x}	sd.
1	8/8	2.29	0.55
2	8/8	2.62	0.57
3	7/7	3.08	0.23
4	8/8	3.28	0.15
5	6/6	3.34	0.13
6	3/3	3.26	0.18
7	3/3	2.94	0.08
8	4/4	2.99	0.11

Table 3. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of febrile reaction ($^{\circ}\text{C}$) in sheep infected with TBF.

Day of reaction	n	\bar{x}	sd.
-1	16	39.4	0.6
1	18	41.1	0.7
2	18	41.3	0.4
3	17	40.8	0.5
4	16	40.5	0.6
5	9	40.4	0.6
6	11	40.4	0.8
7	11	40.1	0.6
8	13	40.0	0.8
9	13	40.0	0.9

Table 4. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of total leucocyte counts ($\times 10^3$ per cu. mm.) in sheep infected with TBF.

Day of reaction	n	\bar{x}	sd
Before inoculation	18	12.7	2.9
-1	15	12.7	3.1
1	18	9.9	2.8
2	18	7.3	1.5
3	18	8.1	1.7
4	17	7.8	1.9
5	10	7.7	1.3
6	11	7.2	1.7
7	12	7.1	1.6
8	12	7.7	2.4
9	12	7.9	3.2

Table 5. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of eosinophil counts ($\times 10^2$ per cu. mm.) in sheep infected with TBF.

Day of reaction	n	\bar{x}	sd
-2	19	7.4	9.8
-1	20	4.5	4.1
1	20	1.7	1.5
2	20	1.0	0.8
3	20	1.2	1.5
4	20	0.4	0.5
5	14	0.6	0.6
6	15	0.4	0.8
7	16	0.6	0.9
8	15	0.2	0.5
9	14	0.3	0.3
10	12	0.6	0.9
11	13	0.5	0.6
12	7	1.5	1.5

Table 6. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of lymphocyte counts ($\times 10^3$ per cu. mm.) in sheep infected with TBF.

Day of reaction	n	\bar{x}	sd
-2	19	7.2	2.4
-1	20	6.6	2.0
1	20	4.3	1.7
2	20	3.0	1.1
3	20	3.5	1.2
4	20	3.6	1.4
5	14	3.9	1.2
6	15	4.4	1.3
7	16	4.5	1.2
8	15	5.4	2.0
9	14	5.5	2.4
10	12	4.8	1.6
11	13	4.9	1.3
12	7	4.5	0.7

Table 7. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of neutrophil counts ($\times 10^3$ per cu. mm.) in sheep infected with TBF.

Day of reaction	n	\bar{x}	sd
-2	18	4.0	2.6
-1	20	4.5	2.7
1	20	4.7	1.8
2	20	3.5	1.1
3	20	3.7	1.4
4	19	3.5	1.8
5	14	2.6	1.1
6	14	2.0	0.7
7	15	1.7	1.0
8	15	1.6	0.9
9	14	1.6	0.8
10	12	1.7	0.6
11	13	1.6	1.0
12	7	1.8	0.9

Table 8. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of monocyte counts ($\times 10^2$ per cu. mm.) in sheep infected with TBF.

Day of reaction	n	\bar{x}	sd
-2	19	3.8	3.3
-1	20	4.5	4.0
1	20	4.4	3.1
2	20	5.7	3.5
3	20	5.3	3.4
4	19	6.1	4.2
5	14	6.2	2.1
6	14	5.2	2.3
7	15	4.8	3.1
8	15	4.0	2.2
9	14	4.5	2.9
10	12	4.8	2.4
11	13	4.3	3.4
12	7	2.4	1.1

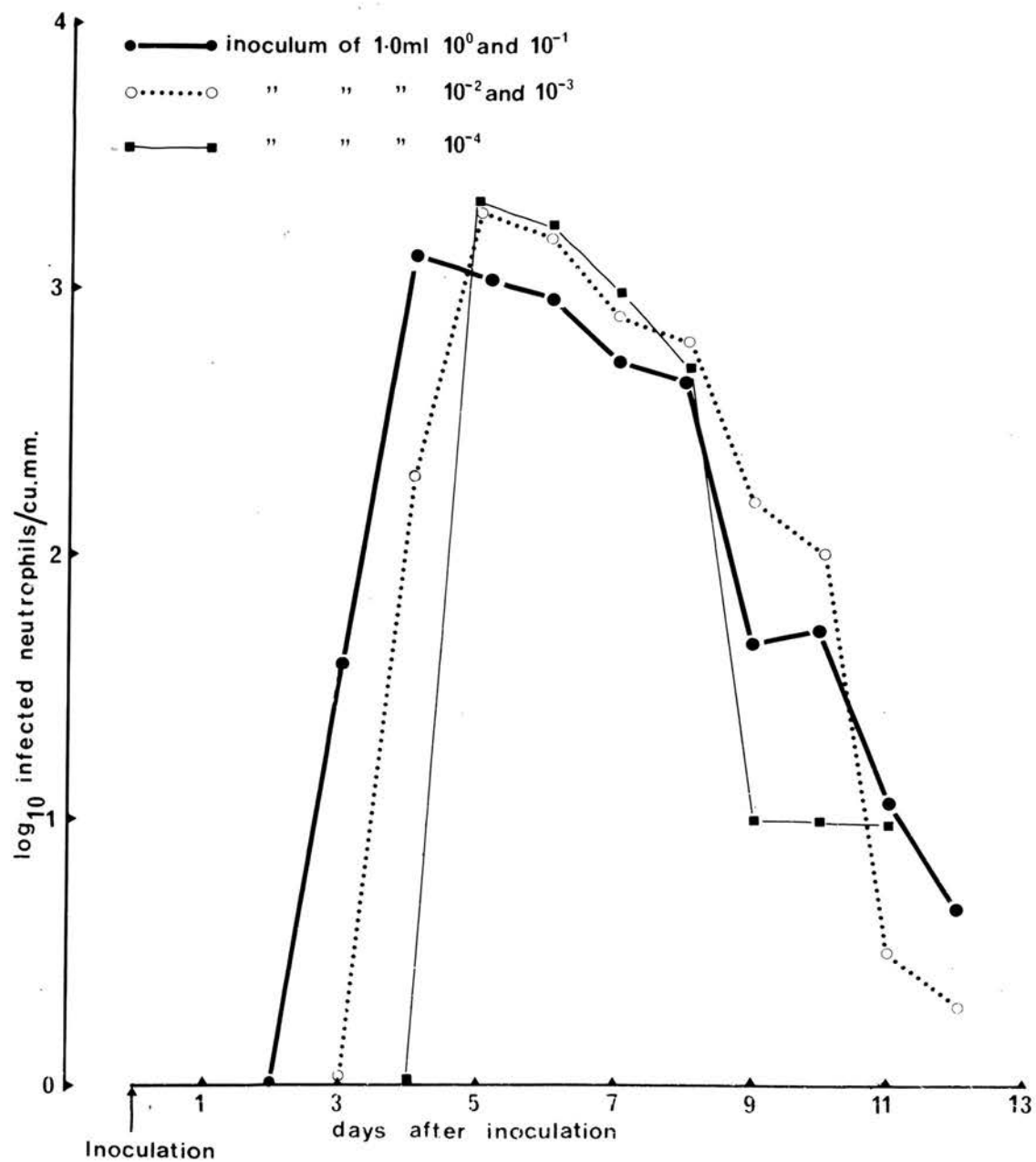


Fig.1 MEAN TBF PARASITAEMIC REACTIONS IN SHEEP AFTER
INOCULATION OF DIFFERENT DOSES

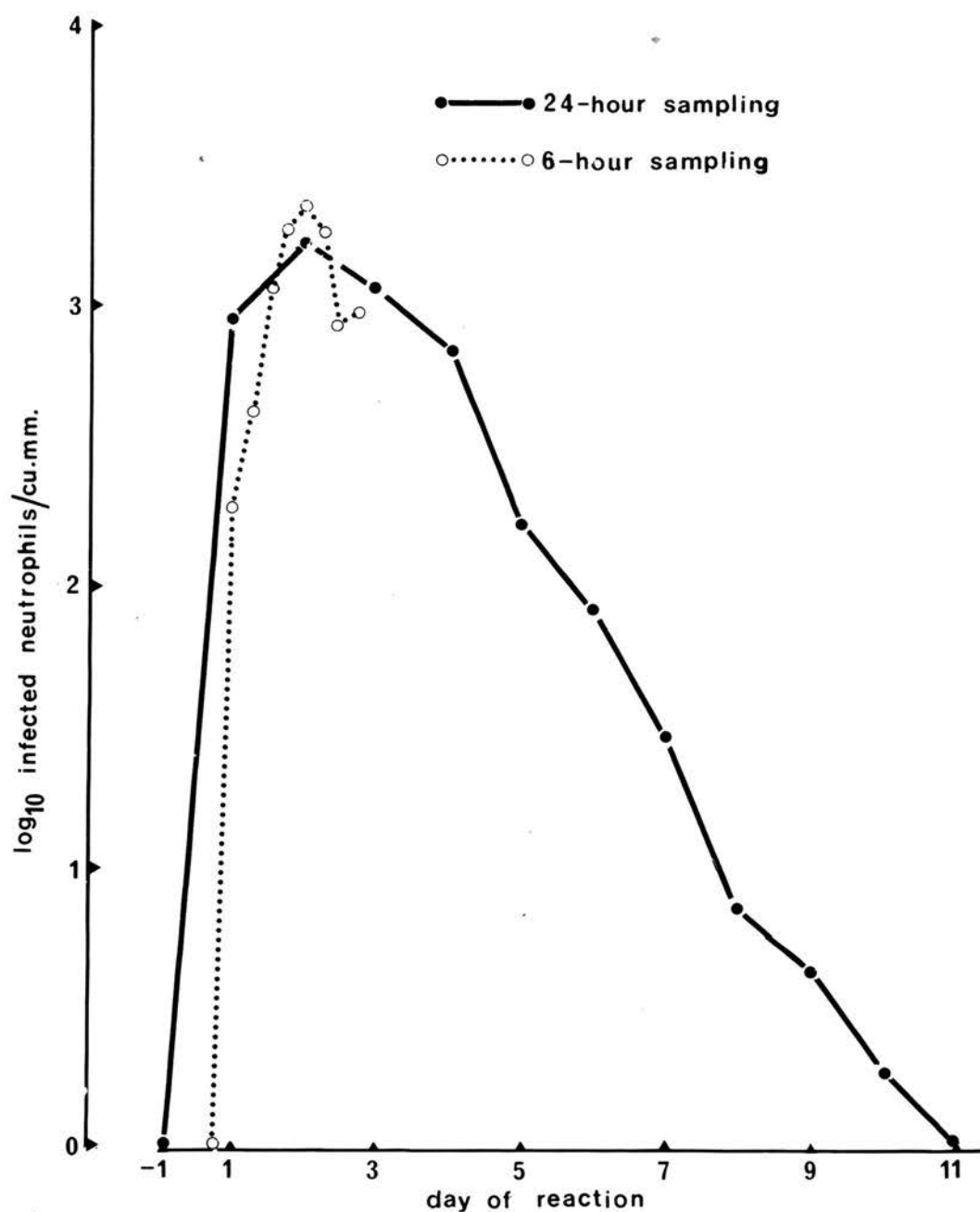


Fig.2 MEAN TBF PARASITAEMIC REACTION
IN SHEEP

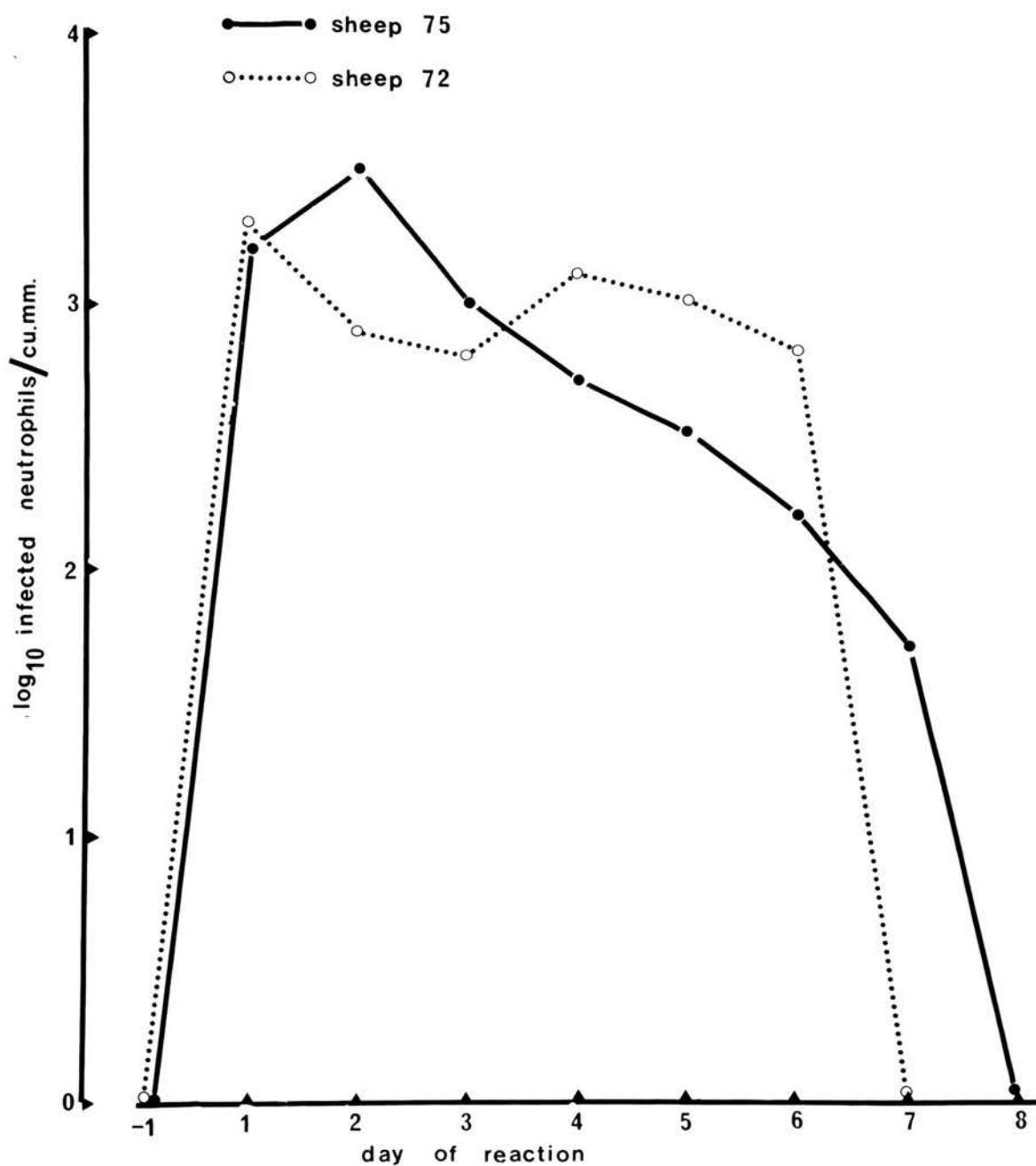


Fig.3 TYPICAL MONOPHASIC AND DIPHASIC PARASITAEMIC REACTIONS IN SHEEP INFECTED WITH TBF

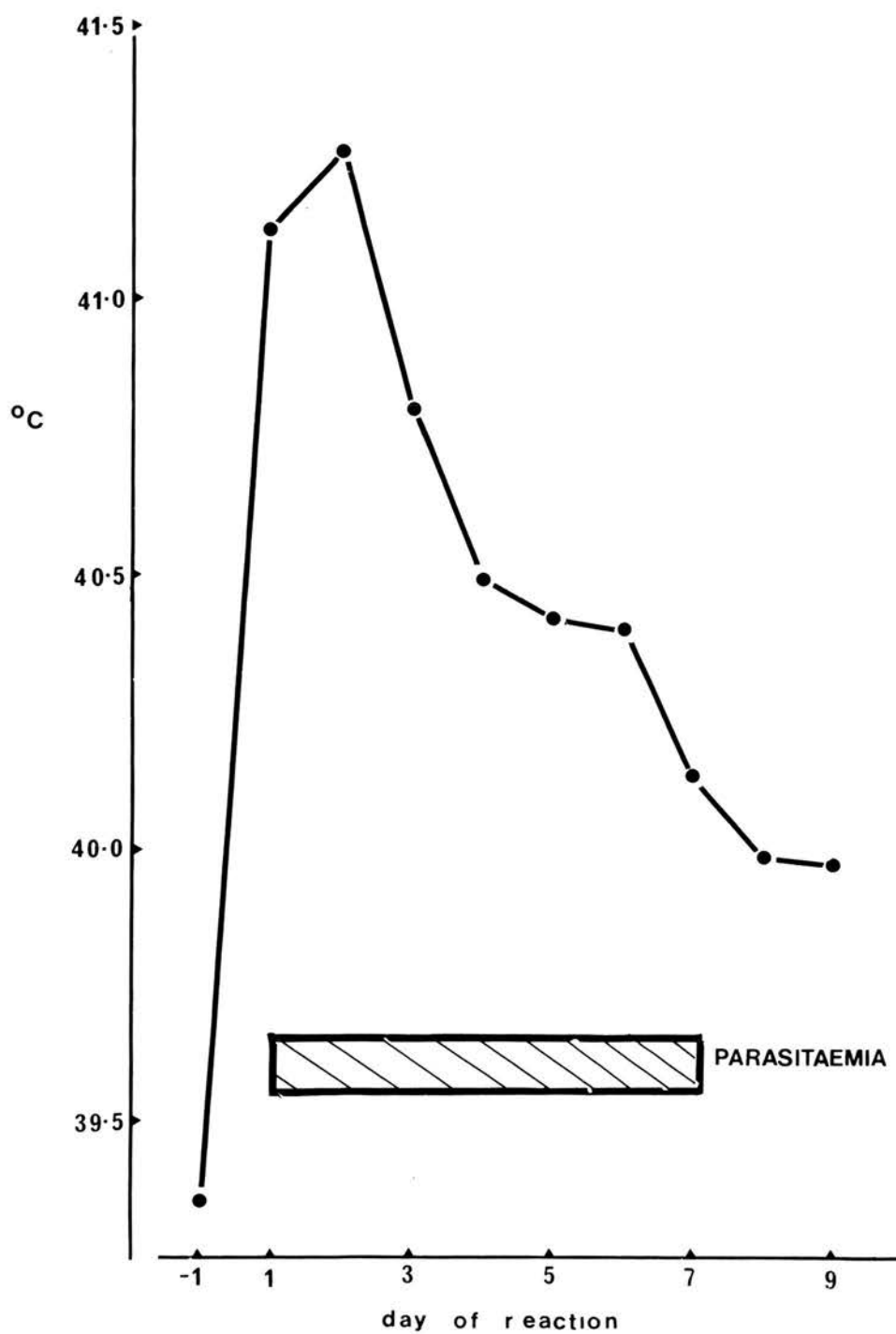


Fig.4 MEAN FEBRILE REACTION OF 18
SHEEP REACTING TO TBF

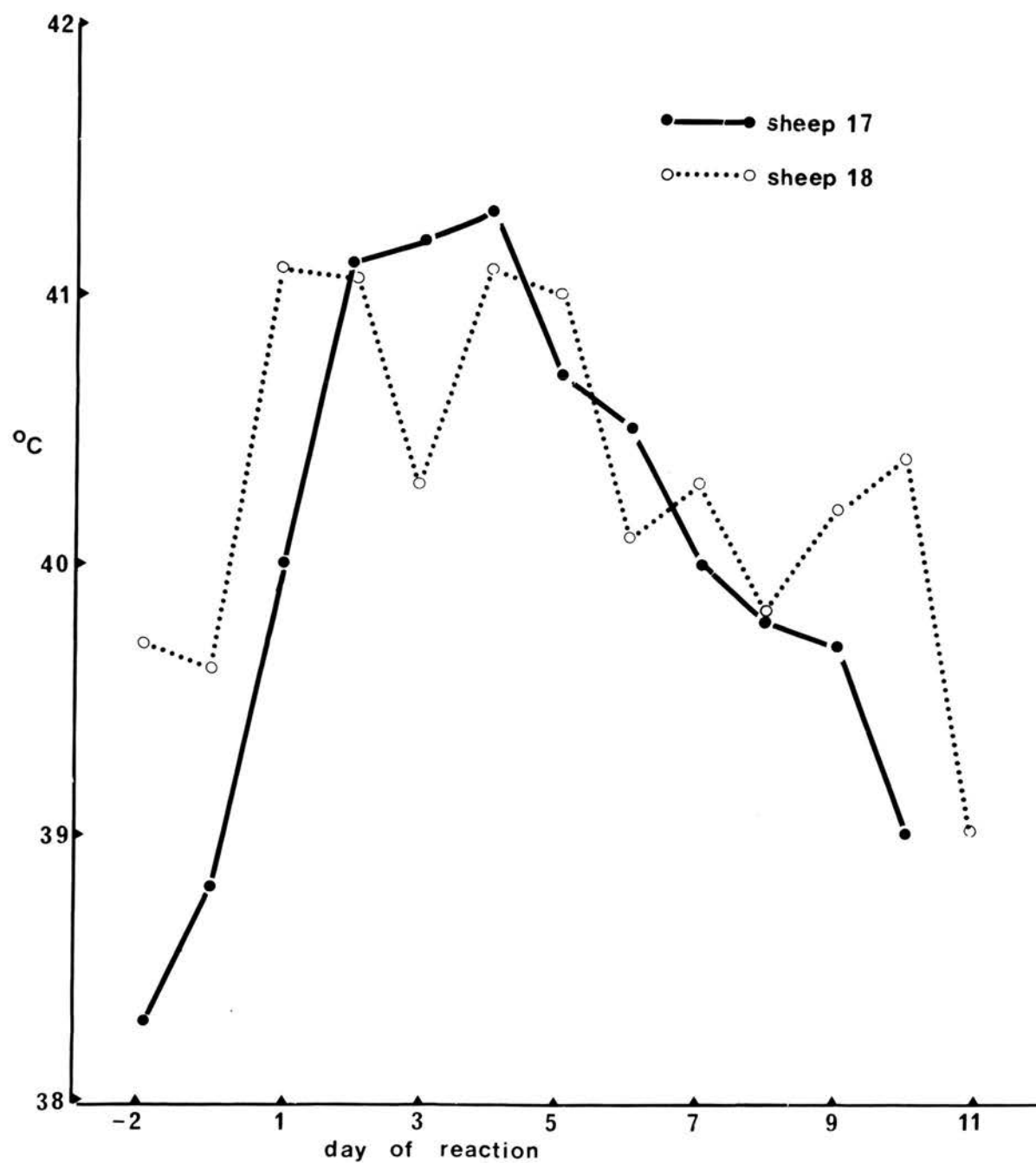


Fig.5 TYPICAL MONOPHASIC AND POLYPHASIC FEBRILE REACTIONS IN SHEEP INFECTED WITH TBF

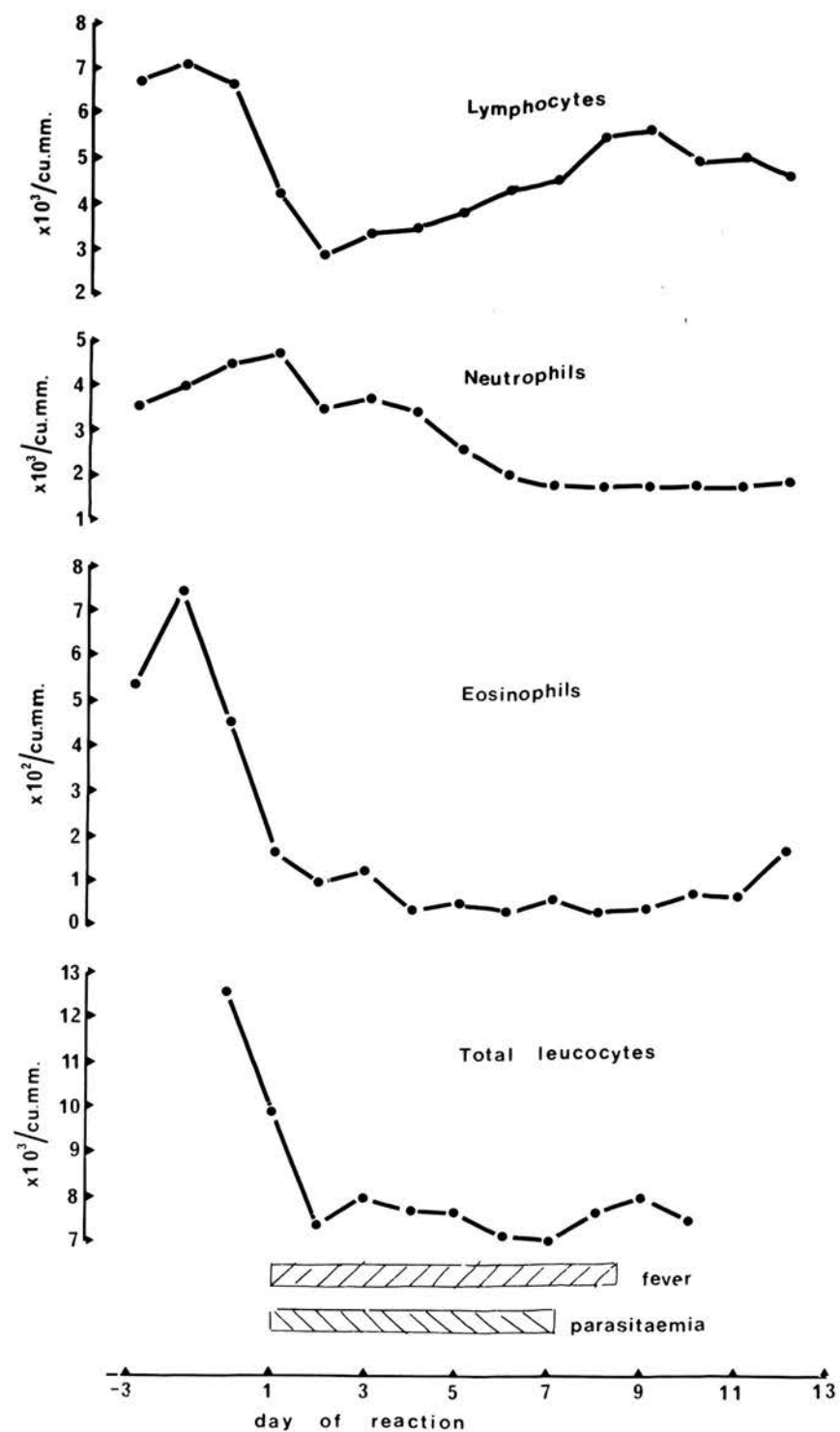


Fig.6 MEAN LEUCOCYTE COUNTS OF 20 SHEEP
REACTING TO TBF

CHAPTER 3DISTRIBUTION OF *C.phagocytophila* IN TISSUES OF INFECTED ANIMALS

It is not known where *C.phagocytophila* multiplies in the infected sheep, or in which tissues other than blood it may be found.

The work recorded in this chapter was intended to investigate methods of studying *C.phagocytophila* distribution, as a guide to future studies on animals infected with BPF.

Materials and methods

1. Animals. Sheep were inoculated with 1.0 ml of a 10^{-1} dilution of infected blood from a lowtemperature storage bank. Thirty-five sheep which were inoculated with this dose of $10^{2.5}$ 50% sheep infective doses (SID_{50}) had a prepatent period of 4.0 ± 0.4 days.

2. Tissue preparation. Eight sheep were killed and exsanguinated at different stages of reaction after inoculation, from 3 days before the expected onset of reaction to the 22nd day of reaction. Tissues were collected aseptically, cleaned of fat and other extraneous tissue, and minced finely with scissors if necessary. The mince was diluted to 10% in PBS, and homogenised for 3 minutes in a blender.¹

The cell suspension was then centrifuged at 1200 g for 10 minutes, and the pellet resuspended in PBS to the equivalent strength of 1 ml suspension from 1 g of tissue. 10% DMSO was added to this suspension, which was then stored at -196°C until use.

The tissues prepared were: lung, thymus, blood, bone marrow,

1. Ato-mix. Measuring and Scientific Equipment, Ltd., London.

spleen pulp, mesenteric lymph node, mediastinal lymph node, pancreas, liver, kidney cortex, adrenal, and myocardium. Blood was collected in EDTA. Bone marrow was obtained by compressing ribs in a vice, and collecting the expressed fluid bone marrow.

3. Tissue inoculation. Tissues after thawing were inoculated to susceptible sheep. For titrations, dilutions were made in PBS, and one sheep inoculated with each dilution. These sheep were then monitored for evidence of TBF infection.

4. Bone marrow biopsies. Serial sternal bone marrow biopsies were taken from 2 sheep during the prepatent period and reaction to TBF, by a method similar to that used by Wilde (1961) in cattle. The sheep were cast, the area over the 3rd and 4th sternebrae clipped and cleaned, and the subcutaneous tissue, muscle, connective tissue, and periosteum anaesthetised with a 3% lignocaine solution. A Salah needle, 40 mm in length, was pushed through the cortex of the 3rd or 4th sternebra into the marrow cavity. A small amount of marrow was withdrawn into a syringe. Smears were made, fixed, stained Giemsa, and examined for the presence of particles with the characteristic morphology of C.phagocytophila.

5. Impression smears. Impression smears from tissues other than bone marrow were also made and examined.

6. Fluroescent antibody studies. Fresh tissues from sheep were frozen in an isopentane-liquid nitrogen mixture at -160°C (Maxwell, Ward, and Nairn, 1966). Sections were cut on a cryostat to a thickness

of 8μ , and attached to clean glass slides. These were either wrapped in foil and stored at -196°C , or fixed and stained for immediate use.

Hyperimmune sera were prepared in 2 recovered sheep by 2 inoculations of autologous infected blood that had been stored. The sheep were bled 1 week after the last inoculation, and sera prepared. The complement fixation test titre of these sera to TBF antigen was 1/256. Hyperimmune and negative sera were conjugated with fluorescein isothiocyanate (FITC) by the method of Nairn (1969). Homogenised sheep liver was prepared and all conjugated sera adsorbed overnight with this at 4°C .

Prior to staining, slides were gradually warmed to room temperature. They were fixed in acetone for 10 minutes at 4°C , air dried, and areas of the slide ringed with nail varnish.

In the indirect fluorescent antibody test (IFAT), serum was placed on the ringed areas of the slide, and incubated in a humidity chamber for 45 minutes at room temperature. The sera were washed from the slides with PBS, and the slides washed twice for 10 minutes in fresh PBS. The slides were then stained with FITC-conjugated rabbit anti-sheep globulin,¹ and incubated and washed as before.

In the direct FAT, conjugated serum was added to the ringed areas of the slide, and incubated and washed as above.

On occasion, after staining the slides were immersed for 2 minutes in 0.1% Evan's Blue, to reduce nonspecific fluorescence.

Parallel sections on the same slide were stained with negative and hyperimmune sera.

The slides were examined with a Nikon fluorescence microscope, under ultra-violet-blue light, using a Toshiba SH200 mercury vapour bulb.

1. Flow Laboratories. Irvine, Scotland.

Results

1. Tissue inoculation. C.phagocytophila was first detected in the lungs of a sheep killed 24 hours after inoculation (Table 9). By 2 days before onset of reaction (i.e. 2 days after inoculation), infectivity was found also in blood and bone marrow, and had spread to most of the other tissues 1 day before onset.

During reaction, highest titres were consistently recorded from lung, followed by thymus and spleen, although blood and bone marrow were not tested in this period. Those tissues with consistently low titres were pancreas, kidney cortex, adrenal, and myocardium. By the 23rd day of the reaction, no infectivity was detected in any tissue.

2. Tissue smears and bone marrow biopsies. No visible evidence of C.phagocytophila infection was detected in any bone marrow biopsy smear.

In other tissues, the organism was seen only in spleen cells and occasional lung macrophages.

3. Fluorescent antibody studies. Examination of sections proved difficult due to the level of nonspecific fluorescence. Lung sections in particular were used, and sections from 2 of 8 infected sheep showed much brighter fluorescence with immune than with control sera. Results were, however, inconsistent and difficult to interpret, and the technique as used was not considered to be a reliable indicator of the presence of C.phagocytophila.

Discussion

Of the 3 methods used to detect the presence of C.phagocytophila in tissues of infected animals, inoculation of tissue suspensions was

the only effective one. The only system available for the inoculation and titration of infectivity involved the use of sheep, and the number of sheep available limited the accuracy by the fact that only one sheep per dilution could be used.

Isolation of C.phagocytophila from the lung within 24 hours of inoculation could be due to persistence of the inoculum. However, the inoculum contained only $10^{2.5} \text{SID}_{50}$, and the lung titre was $10^{0.5} \text{SID}_{50}/\text{g}$, which was almost certainly an underestimate due to the freezing of the organism. Multiplication in the lungs, therefore, seems to have taken place within the 24-hour period.

The subsequent spread of the organism to most other tissues before the onset of visible parasitaemia, indicates that the visible form of C.phagocytophila in leucocytes is not the vehicle by which infectivity is spread throughout the body. Indeed the organism in the leucocytes may be a stage of only minor significance.

The lack of infectivity in the tissues of one sheep by the 23rd day of reaction may not be typical. It is known that not all sheep develop latent infections, and this sheep may have been one in which the organism did not persist. Alternatively, the sample size may not have been adequate to detect low levels of infectivity.

The method used had limitations. Extracellular organisms, or possibly organisms released if any cells were disrupted during preparation, would not be detected but would be left in the supernatant fluid after centrifugation. The fact that high titres were detected in some tissues validated the method to a large extent, although it may have masked the relative infectivities of some tissues.

Cryopreservation was necessary due to the lack of sheep at the time. Some loss of titre is known to occur on freezing, so the

sensitivity of the method to detect low titres was impaired.

While the organism was seen in spleen and lung smears, it was not visible in other tissues, although infectivity was present. It is not known in what cell or in what form it was present in these tissues.

The FAT did not prove of value due to the degree of nonspecific fluorescence. A more specific antiserum, which in turn requires larger amounts of specific antigen, is probably necessary to effectively utilise the FAT.

These investigations gave an indication of probable useful methods of investigation to adopt for BPF.

Table 9. Infectivity titres ($SID_{50/3}$) of tissues of sheep infected with TBF.

Tissue	Day of reaction							
	-3	-2	-1	1	3	4	6	23
Lung	0.5	0.5	3.5	1.5	4.5	3.0	3.5	< 0
Thymus	NT	< 0	0.5	1.5	0.5-3.0	2.5	2.5	< 0
Blood	< 0	1.5	2.5	NT	NT	NT	NT	< 0
Bone marrow	< 0	≥ 0.5	2.5	NT	NT	NT	< 2	< 0
Spleen	NT	< 0	0.5-2.0	≥ 0.5	0.5-3.0	2.5-4.0	≥ 2	< 0
Mesenteric lymph node	< 0	< 0	0.5-2.0	< 0	0.5-3.0	< 2	≥ 2	< 0
Mediastinal lymph node	< 0	< 0	0.5-2.0	< 0	0.5-3.0	< 2	≥ 2	< 0
Liver	NT	< 0	< 2.0	< 0	0.5-3.0	≥ 2	< 2	< 0

NT - not tested.

CHAPTER 4SEROLOGICAL STUDIES

The presence of specific antibodies to TBF has been demonstrated by Tuomi (1967d), but no serological test is used routinely.

Materials and Methods1. Complement Fixation Test (CFT)

a) Antigen production. The method used was that described briefly by Snodgrass and Ramachandran (1971) (Appendix 1). Blood was collected from parasitaemic sheep into Alsever's solution, containing sufficient additives to give final concentrations of approximately 20 u/ml heparin, 100 iu/ml penicillin, 100 μ g/ml streptomycin, and 5 μ g/ml fungizone.¹ Up to 300 mls blood was collected from the live animal. If greater volumes were required, the animal was killed and exsanguinated.

The blood was centrifuged at 1250 g, and the buffy coat collected. This was repeated several times, to remove as many erythrocytes as possible. Sterile distilled water was then added to the buffy coat to lyse erythrocytes. After 30 seconds a much larger volume of PBS was added to restore isotonicity. The leucocytes were washed twice in PBS to remove all haemoglobin and soluble erythrocyte antigen, and then suspended in PBS or Hank's balanced salt solution, in a volume equal to one-hundredth of the original blood volume.

The leucocytes were disrupted either by 3 minutes sonication,² or by 3 cycles of alternate freezing and thawing. The suspension was

1. E.R. Squibb & Sons, Liverpool.

2. Ultrasonic Disintegrator. Measuring and Scientific Equipment, London.

then centrifuged at 1250 g for 15 minutes, and the supernatant fluid kept as the antigenic fraction.

Spleen, lung, and occasionally other tissues, were homogenised briefly to liberate the cells, and thereafter treated in the same way as leucocytes.

All antigens were stored in small aliquots at -20°C without a preservative.

Anticomplementary activity was removed by absorption overnight at 4°C with heat-inactivated complement (Ross, C.A.C., personal communication).

b) Sera. Fourteen sheep were bled at intervals for up to 48 weeks after recovery from initial infection, and serum prepared. Twelve of these sheep were then challenged with TBF, and bled for serum at intervals for a further period of 24 days.

Sera were stored at -20°C .

c) Technique. The technique detailed in Grist, Ross, Bell, and Stott (1966) was followed.

2. Immunodiffusion test (IDT). Antigens and antisera were as used for the CFT. An antiserum with a high CF titre was concentrated to one-fifth of its original volume by dialysis against carbowax,¹ and subsequently against PBS.

One per cent Ionagar No.2² was used as gel. Plates were placed in a humid atmosphere at room temperature, and read after 48 hours.

1. George T. Gurr Ltd. Bucks.

2. Oxoid Ltd. London.

3. Fluorescent antibody test (FAT). Antigen consisted of blood or buffy coat smears from parasitaemic sheep. Sera were as used for the CFT. Other details are those previously described in chapter 3.

Results

1. CFT.

a) Demonstration of antigen. Antigen was successfully produced from 11 sheep initially (Snodgrass and Ramachandran, 1971). The specificity of the reactions was confirmed by testing, against allogeneic and autologous antigens, preinoculation and hyperimmune sera from 2 sheep that were hyperimmunised by inoculation of their own infected blood that had been stored at low temperature. Antibody titres to the two antigens were similar, which confirmed that the reaction was specific, and not due to interference from nonspecific factors in the antigen.

Antigen preparations were made from a total of 21 sheep. The comparative figures for parasitaemia percentage and antigen titre are given in Table 10, for the 10 sheep for which both figures are known. The percentage of infected neutrophils ranged from 10 to 86, and in general, sheep with greater parasitaemias gave higher yields of antigen. The correlation, however, was not significant ($r = 0.54$, d.f.8, $p > 0.05$). Antigen was not demonstrable in 2 sheep in which the percentage of infected neutrophils was 10 and 14 respectively. Blood was usually taken for antigen preparation when parasitaemia was at its highest i.e. on the first or second days of reaction.

Fresh antigen preparations had a high infectivity titre when inoculated to sheep.

The antigen was satisfactorily stored at -20°C for 10 weeks, the longest period tested, but repeated freezing and thawing reduced activity. The antigen was labile to heating at 56°C for 30 minutes.

Attempts were made to improve antigen yield by improving separation of leucocytes from erythrocytes. These methods involve agglutination and sedimentation of erythrocytes, and the methods of Skoog and Beck (1956), Boyum (1968), and Otto (1970), were tried. They were not found to be suitable for sheep blood.

Antigen preparations from tissues other than leucocytes gave occasionally positive but inconsistent results.

b) Demonstration of antibody. Antibody titres are expressed as \log_2 of the reciprocal of the CF titre. Mean titre before inoculation was 3.4 ± 0.9 , sera from 2 sheep showing a low level of nonspecific complement fixation (Fig. 7, Table 11). Antibodies appeared in all sheep except one between days 11 and 14. Mean peak antibody titre was 8.6 ± 2.1 . Titres thereafter dropped gradually over 6 months, by which time they were similar to preinoculation levels (Fig. 8).

Considerable variation occurred within this antibody response. One animal showed a low positive titre from the 6th - 11th weeks only, while others maintained titres near the maximum for nearly 6 months.

Twelve sheep were challenged 10 months after primary reaction. None showed any fever or other clinical sign, and parasitaemia was detected in one animal on one day only. All except two of these sheep showed a rise in CF antibodies between days 7 and 13, from a level of 4.2 ± 1.4 before inoculation, to 6.3 ± 2.1 on day 13 (Table 12). The peak titre after challenge was lower than after primary reaction, but not significantly so ($t = 2.02$, $p > 0.05$) (Fig. 7).

Two immune sera taken 14 days after inoculation were fractionated on a Sephadex G-200¹ column. CF antibodies to TBF were found mostly in the second eluent^{ate} peak, which was identified as IgG by immunoelectrophoresis, and some in the first peak, which was identified as IgM.

2. IDT. No lines of precipitation formed between antigens from 8 sheep and antisera that reacted in the CFT. The concentrated immune serum formed precipitation lines with positive antigens, but this was not consistently reproducible.

3. FAT. Fluorescent particles with the morphology of C.phagocytophila were seen in the cytoplasm of the neutrophils, as described by Tuomi (1967d). Their identity with C.phagocytophila was demonstrated by noting certain fluorescent particles, fixing the slides with methanol and counterstaining Giemsa, and observing the same particles under light microscopy.

Fluorescence was not bright, and results were not consistent.

Discussion

The CFT for TBF proved a reliable method for detecting serological response after infection.

The antigen titre was not found to be related to the percentage of infected neutrophils. A quantitative estimate of the number of infected neutrophils is more accurate, and it might have been possible to show a relationship between this figure and antigen titre. However, the data were not available.

The method of antigen production was not ideal. Only low yields were obtained, antigen from one sheep being sufficient to test no more

1. Pharmacia (GB) Ltd. London.

than 100 sera. It is known that infectivity titres in the blood are not as high as in some other tissues (chapter 3), and it may be that not enough organisms were present. The antigen production method used was not suitable for tissues other than blood.

Attempts at erythrocyte sedimentation techniques to improve the leucocyte yield were not successful. Ovine red cells do not sediment very readily (Schalm, 1965), so techniques based on sedimentation are unlikely to be satisfactory.

The period of visible parasitaemia had finished in most sheep by the time that CF antibodies were detected, so these antibodies were not necessary to overcome the infection. Ten months after initial infection, CF antibodies were absent, though immunity was complete. This suggests that the presence of these antibodies was not related to immunity, but was merely an indication of previous TBF infection. Snodgrass and Ramachandran (1971) thought CF antibodies probably were related to immunity, but they had less data available.

After challenge, the animals did not show a secondary response, which would have involved much quicker production of antibody to higher levels. This may have been due to the interval after the primary sensitisation being too long, or to slight antigenic change in the organism.

The test was not used on sera for diagnosis. If crossreaction occurred between different C.phagocytophila isolates, as it has been shown to do by Tuomi (1967d) in the FAT, the CFT would be a useful diagnostic tool.

Improved serological methods probably require methods of in vitro culture to produce antigen.

Whereas CF antigens can be present in crude tissue extracts, the

IDT is not such a sensitive technique. IDT antigen requires to have soluble products which can diffuse through the gel. This is probably the reason that the CFT reagents did not react in an IDT.

The FAT applied to leucocyte preparations is difficult to interpret for reasons of nonspecific fluorescence of the leucocytes (Löhr and Ross, 1969). It did not prove suitable in this instance for use as a routine serological technique (see also chapter 14).

Table 10. Relationship of visible parasitaemia to CF antigen titre.

Sheep No.	Parasitaemia (%)	Antigen titre
Y2	10	< 1/2
Y4	14	< 1/2
Y36	15	1/8
B94	26	1/16
Y128	36	1/64
Y94	48	1/16
Y144	50	1/128
B86	60	1/4
38	80	1/128
Y29	86	1/16

$r = 0.54$; d.f. = 8; $p > 0.05$.



Table 11. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of CF serum titres (\log_2 reciprocal) in sheep infected with TBF.

Weeks after Inoculation	n	\bar{x}	sd
Before inoculation	14	3.0	0
1	5	3.0	0
1.5	5	3.2	0.3
22	12	8.3	2.0
2.5	7	7.9	2.5
3	8	6.9	1.7
4	14	7.2	2.2
6	8	7.9	1.2
8	11	7.2	1.3
9-12	10	6.2	0.9
13-16	14	5.6	1.4
17-20	12	4.9	1.3
21-26	12	4.5	1.6
30-48	8	4.4	1.1

Table 12. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of CF serum titres (\log_2 reciprocal) in sheep challenged with TBF.

Days after challenge	n	\bar{x}	sd
0	11	4.2	1.4
4	12	4.0	1.2
7	12	4.0	1.3
13	12	6.3	2.1
24	10	5.4	1.8

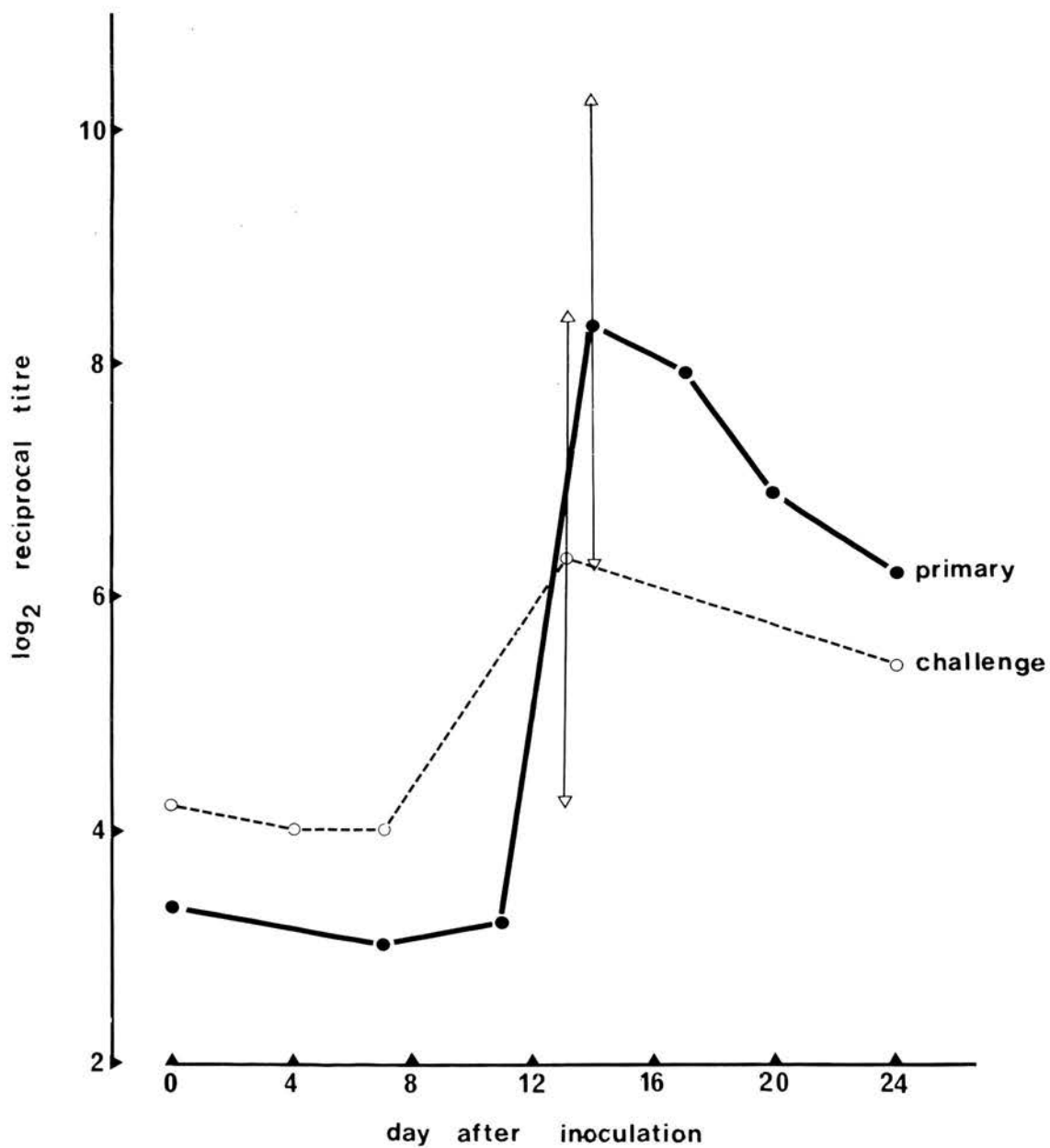


Fig.7 CF SERUM TITRES (MEAN \pm SD) OF SHEEP
AFTER PRIMARY AND CHALLENGE
INOCULATIONS WITH TBF

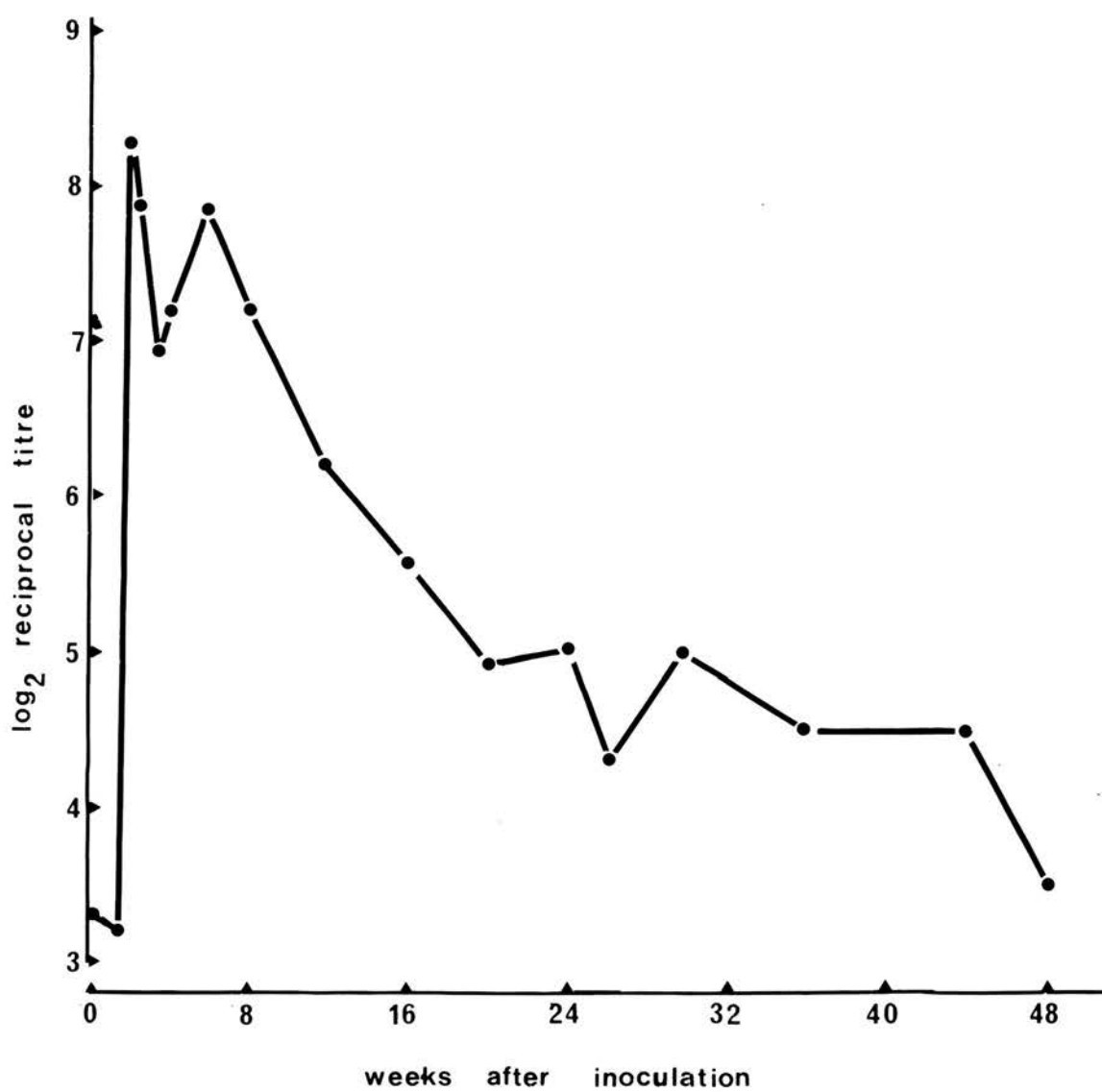


Fig.8 MEAN CF SERUM TITRE OF 14 SHEEP
AFTER PRIMARY TBF REACTION

CHAPTER 5ATTEMPTED PROPAGATION OF *C.phagocytophila* IN CELL AND TISSUE CULTURE

Attempts to cultivate *C.phagocytophila* in eggs (Foggie, 1951; Hudson, 1950) and unspecified tissue culture systems (Tuomi, 1967a), have failed.

This chapter records the methods used to attempt the cultivation of the organism by two different methods.

Materials and methods

1. Leucocyte culture. The method adopted was similar to that used by Tokuda, Fukusho, Morimoto, and Watanabe (1962) to culture bovine leucocytes. Blood from normal and TBF-infected sheep was collected in heparin, and the whole blood distributed in 0.5 ml volumes to culture tubes with coverslips. These were incubated at 37°C. After 3 days, the plasma, erythrocytes, and unattached leucocytes were washed off with PBS, and replaced with autologous heat-inactivated serum.

2. Rhipicephalus appendiculatus cultures. Cultures of explants of all tissues of the developing adult tick *R.appendiculatus* from nymphs 8 days after engorgment were received from Miss I. Cunningham. Three 9-day-old and three 17-day-old cultures were inoculated each with 1 ml of infected buffy coat suspension in growth medium. This was washed off after 24 hours and replaced with fresh growth medium.

The growth medium with foetal bovine serum was one developed by Miss Cunningham. Incubation was at 28°C.

3. Infectivity of cultures. At intervals after initiation of each culture, both cells and medium were inoculated to susceptible sheep. These were then monitored for evidence of BPF infection.

4. Examination of cultures. Coverslips were removed, fixed, and stained with Giemsa's stain, to observe the progress of the leucocyte cultures. The tick cultures were examined alive for signs of a healthy culture e.g. peristalsis.

Results

1. Leucocyte cultures. The cultures developed in a similar manner to that described by Tokuda et al (1962). Neutrophils attached for a few days only, and thereafter only mononuclear cells and later multinucleated cells occurred. It was found that infected neutrophils and monocytes did not attach to the glass at all, but remained in the medium, where they could be detected for up to 24 hours after inoculation.

Infectivity of the cultures from infected sheep was tested between 3 and 8 days after initiation, and no evidence of infection was obtained.

2. R.appendiculatus cultures. The cultures remained healthy. All media and tissues 22 days after attempted infection were inoculated to sheep. No infectivity was detected.

Discussion

The leucocyte culture method used was suitable only for mononuclear cells, as in control cultures neutrophils attached for up to 4 days only. Neutrophils and monocytes infected with C.phagocytophila did

not attach at all. This may be an impairment of neutrophil behaviour, as noted by Foster and Cameron (1970) in a study of C.phagocytophila-infected neutrophils.

Only a few tick cultures were available for use. The method as used did not succeed in propagating C.phagocytophila but no variation of technique was tried.

R.appendiculatus cultures were available, but Ixodes ricinus, the principal TBF vector in U.K. (MacLeod and Gordon, 1933), would have been preferable. There are, however, serious difficulties introduced by the latter's diapause-regulated life-cycle when it is used as a vector in the laboratory.

An attempted nymph-adult transmission of TBF by R.appendiculatus did not succeed, which confirms the unsuitability of this tick.

CHAPTER 6ATTEMPTED INFECTION OF MICE WITH C.phagocytophila

Foggie and Hood (1961) reported the adaptation of strains of C.phagocytophila to splenectomised guinea-pigs, normal guinea-pigs, and splenectomised mice. These strains produced mild disease, and were still capable of producing TBF in sheep.

I evaluated the effect of immunosuppressants on mice, to ascertain whether the immunosuppressed mouse could be used to assay ovine C.phagocytophila.

Materials and methods

1. Mice. Female Theiler's original mice¹ were used. Their weights ranged from 20-30 grams, and their ages from 6-10 weeks.

2. Immunosuppressants. Betamethasone² was inoculated to 48 mice intraperitoneally for 5 days, at a daily dose of 10 mg/Kg. Twenty-eight of these mice were inoculated with C.phagocytophila on the 5th day.

Cyclophosphamide³ was inoculated to 16 mice by the intraperitoneal route at a dose of 50 mg/Kg daily for 4 days. Eight of these mice were inoculated with TBF on the first day.

Eight mice were inoculated with C.phagocytophila alone.

3. Inoculation. Fresh or thawed sheep blood was inoculated to the mice in 0.25 ml volumes. Half of each group was inoculated by

-
1. A. Tuck & Sons. Rayleigh, Essex.
 2. Betsolan. Glaxo, Greenford, England.
 3. Endoxana. Ward, Blenkinsop & Co. Ltd., London.

the intravenous and half by the intraperitoneal route. The infectivity of each inoculum was verified by sheep inoculation.

4. Observations. Blood smears were made from the tail tip of mice every 2nd day. They were fixed, stained Giemsa, and examined for organisms with the morphology of C.phagocytophila. Differential white cell counts were also carried out.

To collect blood, animals were anaesthetised with ether, blood collected from the axillary region, and the mice killed. Blood was collected in heparin from 12 animals, and inoculated to sheep to test for infectivity. From 56 others, serum was prepared, and tested in the CFT with TBF antigen.

Results

1. Examination of smears. Absolute values were not available for the differential leucocyte counts, but percentage differential counts remained similar between infected and control mice.

Single or multiple bodies stained similarly to C.phagocytophila were observed in 13 of 28 mice given betamethasone and TBF; in 6 of 24 mice given betamethasone alone; but not in any other group. The inclusions appeared like the small particles of C.phagocytophila described by Tuomi and von Bonsdorff (1966) (Figs. 9 & 10). Morulae were not seen.

2. Serology. Sera were taken from 2-4 weeks after inoculation, and pooled in groups of 4. All 14 pools tested had CF antibody titres of less than 1/8.

3. Infectivity. Twelve mice treated with betamethasone and inoculated with TBF were killed in groups of 4 at 2, 3, and 4 days after infection. The blood from each group was pooled, and inoculated to sheep. Only the blood taken 2 days after infection was still infective.

Discussion

The failure of C.phagocytophila to multiply in the mice was evidenced by lack of infectivity, absence of typical inclusions in neutrophils, and negative serum antibody results. Foggie and Hood (1961) found that splenectomy was more efficient than cortisone or nitrogen mustard treatment at suppressing resistance to multiplication of the organism.

The nature of the particles seen in the neutrophil cytoplasm of those animals treated with betamethasone is not known. Foggie (1951) reported the appearance in mice of bodies bearing some resemblance to C.phagocytophila in phagocytes, and postulated the presence of a latent rickettsial disease. Foggie and Hood (1961) in their study of the mouse-adapted strain found multiple inclusion bodies, but few morulae. The small particles in their photograph of guinea-pig blood are very similar to those observed in mice in this work.

The particles may be a rickettsia-like organism latent in mice, that became patent after betamethasone immunosuppression. Tyzzer (1938) recorded an agent with similar morphology, occurring in granulocytes, isolated from voles in Massachusetts. This agent, which he named Cytoecetes microti, was transmissible to several other species of rodents. It is possible that the rickettsia-like organisms found

in this work may be similar to Tyzzer's vole agent, and to that observed by Foggie (1951). A similar organism may also have interfered with the work of Foggie and Hood (1961), as they found mainly small particles and few morulae in the rodents.

The presence of these organisms makes mice unsuitable for use as an assay system for C.phagocytophila.

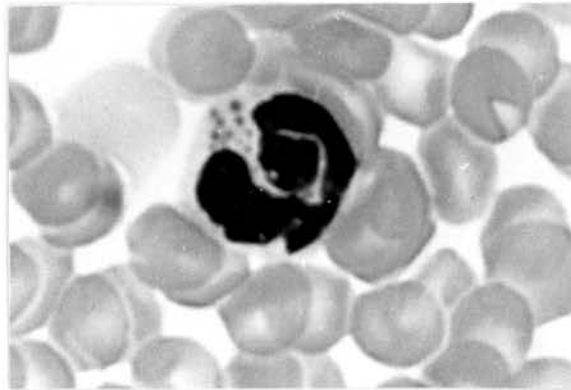


Fig.9 Neutrophil of a mouse injected with betamethasone before inoculation with TBF, showing intracytoplasmic inclusions. X1800.

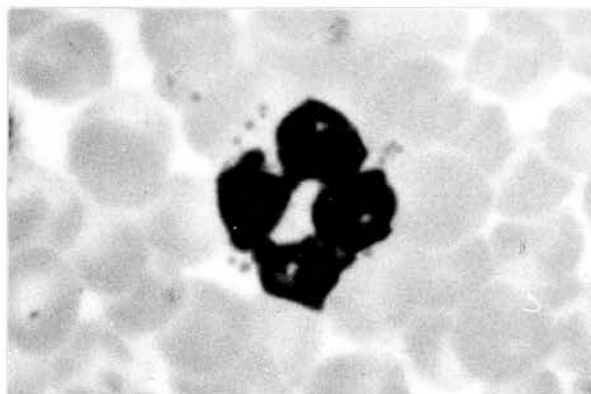


Fig.10 Neutrophil of a mouse injected with betamethasone, showing intracytoplasmic inclusions. X1800.

STUDIES ON BOVINE PETECHIAL FEVER

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CHAPTER 7GENERAL MATERIALS AND METHODS

1. Animals. Unless otherwise stated, the cattle used were Sahiwals crossed with European dairy breeds, from the veterinary department farm at Machakos, Eastern Province, Kenya. This is an area of altitude less than 5000 feet, where BPF is thought not to occur. Females or castrated males were used, in an age range of from 9 months to 2 years. These animals were found to be uniformly susceptible.

Sheep were Hampshire Down or Merino crosses from Molo, in Rift Valley Province. BPF occurs in the Molo area, but not on the farms that supplied the sheep. Females or castrated males were normally about one year old, although older sheep were used on occasion. These animals also were uniformly susceptible.

Animals were usually housed throughout the experimental period. No tick control was practiced on housed animals, but only one case of intercurrent tickborne disease (anaplasmosis) occurred. No BPF has occurred naturally at Kabete, and it was not considered that the animals risked natural BPF.

2. C.ondiri. Various strains of C.ondiri from field cases of BPF were used. The two most frequently used were:

- a) Naivasha ovine (N/O) strain, which was isolated from blood of cattle at Naivasha, and subsequently maintained by passage in sheep and periods of lowtemperature storage. This strain readily infected both cattle and sheep.

- b) Naivasha bovine (N/B) strain, which was isolated from Naivasha, and subsequently maintained by passage in cattle, and periods of low-temperature storage. Although pathogenic for cattle, this strain did not regularly infect sheep.

Other strains were used where stated.

3. Infection. Blood or other tissues from an infected animal were used as inocula. Cattle were usually inoculated intravenously with the buffy coat fraction from 200 ml of blood, although smaller volumes were used on occasion. Sheep were inoculated with whole blood intravenously, volumes of 1 ml or less being sufficient.

4. Observations. Initially, rectal temperatures and blood samples in EDTA were taken daily after inoculation from all animals. However, it became apparent that with the laboratory strains, visible parasitaemia in both sheep and cattle was always associated with a febrile reaction in excess of 40°C (Chapter 10). Thereafter animals were bled only when they showed such a febrile reaction, unless a quantitative result was desired, or unless a field strain not adapted to laboratory passage was being used.

Blood smears stained with Giemsa stain were examined under a X100 oil immersion lens by light microscopy for the presence in the granulocytes or monocytes of C.ondiri (Krauss et al, 1972).

The presence of an inclusion body with the typical structure of C.ondiri was taken as evidence of infection. Before recording a sample as negative, at least 100 neutrophils were examined.

5. Cryopreservation. The method described for low-temperature storage of TBF (chapter 1) was applied to BPF. Sheep blood was stored in 1 ml amounts in sealed glass ampoules. Cattle blood was stored as buffy coat cells from 200 mls blood, the final volume of approximately 2 mls being stored in 5 ml sealed glass ampoules. All blood was stored at -196°C .

This method proved satisfactory for the storage of various strains of BPF, for periods of up to 10 months, the longest interval tested.

Other materials and methods are described in the relevant chapters.

CHAPTER 8INFECTION OF CATTLE OF DIFFERENT BREEDS

Only exotic breeds of cattle usually contract clinical BPF (Haig, 1966). There is no published information on the susceptibility of different exotic breeds to natural or experimental BPF. It was not planned to investigate possible differences, but failure to infect certain experimental cattle compelled rationalisation of their breeds and origins.

Materials and Methods

1. Animals. In addition to Sahiwal crosses from Machakos, the following animals were used:

- a) 15 Herefords from the veterinary department farm at Kabete. No cases of BPF have ever been recorded from this farm.
- b) 3 Sahiwal crosses from Kabete.
- c) 1 Sahiwal cross from Naivasha, an area where BPF is common.
- d) 5 Ayrshires from Machakos.
- e) 3 Boran crosses from Nanyuki, also an area where BPF is common.

Animals were females or castrated males, and were from 9 months to 2 years old.

2. C.ondiri. In addition to N/B and N/O strains, others from Ol Magogo (near Naivasha), Subukia, Mau Narok and Molo were used. Blood

from reacting cattle and sheep was used as inoculum.

Nonreacting cattle were considered to have received a viable inoculum when either a) susceptible controls receiving the same inoculum reacted, or b) they received 200 ml of blood from a parasitaemic animal, a dose which experience showed was always sufficient to cause BPF in a susceptible animal.

Results

A total of 98 cattle received viable inoculations of various strains, and their susceptibility is recorded in Table 13.

Seventy-three of 75 Sahiwal crosses of all origins proved susceptible.

Smaller numbers of other breeds gave different results. While 3 Borans all contracted BPF, 5 Ayrshires and 15 Herefords were found to be resistant.

Discussion

The breeds fell into two categories, those in which the animals were almost wholly susceptible, and those where the animals were apparently resistant. Possible explanations for resistance are the existence of acquired immunity through previous BPF exposure, or the presence of an innate breed resistance.

Acquired immunity is difficult to reconcile with the known susceptibility of animals of other breeds from the same farms of origin. BPF can occur on very localised areas within farms, but Machakos is distant from any known BPF area. Another argument against acquired immunity is provided by the serum titres of the cattle, from complement fixation test with BPF antigen (Chapter 14).

Four Ayrshire cattle had a mean reciprocal preinoculation titre of 24, which was not significantly different from that of the susceptible Sahiwal crosses ($t = 0.60$, d.f. 10). However, it is unlikely that the serum titre is related to immunity.

Breeds present in enzootic areas of Kenya for many generations may have developed a degree of innate resistance to BPF, while comparatively recent importations such as the Sahiwals (Faulkner and Brown, 1953) may be expected to be more susceptible. This view is difficult to reconcile with the field disease, where Ayrshire cattle are commonly affected, and cases have also occurred in Herefords.

These results are an enigma. However, their recognition made subsequent work much simpler, as a ready source of susceptible cattle was available in the Sahiwal crosses. They may also explain the difficulties encountered by earlier workers in maintaining bovine passage series (Piercy, 1953; Kenya, 1957; Kenya, 1960). Danskin and Burdin (1963) found that of over 1000 animals used in transmission experiments, only about half reacted.

Table 13. Susceptibility of cattle of different breeds and origins to BPF.

Breed	Sahiwal			Ayrshire	Boran	Hereford
Origin	Machakos	Kabete	Naivasha	Machakos	Nanyuki	Kabete
No.inoculated	71	3	1	5	3	15
No. reacted	69	3	1	0	3	0
Susceptibility (%)	97	100	100	0	100	0

CHAPTER 9CLINICAL SIGNS

Accounts of the clinical signs of BPF have been given by Piercy (1953), Danskin and Burdin (1963), and Haig (1966). However, there is no record of quantitative details of clinical signs on a large group of experimental animals.

Materials and Methods

1. Animals and C.ondiri. Daily observations were made on a group of 21 Sahiwal crosses from Machakos infected with the N/B strain of BPF. Visits were also made to naturally-occurring field cases. Experimental sheep were infected with various strains.

2. Observations. In addition to the routine microscopical examination for parasitaemia, cattle were examined daily to note general appearance, and in particular the mucosae in the following sites were examined for haemorrhages - vulva-vagina, conjunctiva, labial surface of the gums, and ventral surface of the tongue.

Results.

Observations on field cases did not differ from those described elsewhere.

The twenty-one experimental cattle reacted to BPF with fever and visible parasitaemia. The incubation period was in the range 4 to 10 days. Dullness became apparent on the second or third day of reaction. This was evidenced by a reluctance to rise or move, by

weakness, and by drooping of the head, and was nearly always accompanied by a harsh, staring coat. Thirteen of 21 cattle (62%) showed these symptoms, including all the cattle in the later passage levels. Dullness lasted for 1-6 days.

Fifteen of 21 animals (71%) developed visible haemorrhages. Of the 6 which showed no petechiae, 4 were in the first 3 passages after primary isolation or after a period of low-temperature storage.

The mean appearance of haemorrhages was on day 2.4 ± 0.6 , never on the first day of reaction. They first appeared as very small petechiae, which gradually became larger over 2-3 days. Sometimes the congested background noted by Plowright (1962) was evident, particularly in the fatal cases. Haemorrhages regressed slowly over several days.

Increased intensity of petechiation greatly aided observation. Each site was scored daily for intensity on the following subjective scale:

- 0 petechiae not seen
- 1+ petechiae just visible
- 2+ petechiae easy to see
- 3+ petechiae abundant

The results for the presence or absence of petechiae at various sites for individual animals are given in Table 14, omitting the 9 males and the 7 with pigmented gums from the appropriate observations. The table also gives an intensity score, being a mean of the maximum intensity scored for each individual at that site.

The difference in intensity between the various sites was not significant ($F = 1.31$; d.f. 3, 40).

'Poached egg eyes' were seen in both fatal cases (Fig. 11). A lesser degree of conjunctival oedema was noted in 3 other cases,

accompanied by excessive lachrymation.

One animal developed marked generalised muscular tremors.

Lymph node enlargement was not noted clinically.

Two cattle died of acute BPF i.e. 10%. They died on the second and fourth days of reaction, both with respiratory distress. They showed the same symptoms as the mildly-ill cattle, although to a greater degree.

Sheep were very little affected, although they showed high fever. Some became dull, and showed a much increased respiration rate which continued for a day or two after the temperature had returned to normal.

Discussion

Apart from the absence of palpable lymph node enlargement, the clinical picture observed in the experimental animals was similar to that reported for field cases, although more detailed and sequential observations were possible.

The absence of haemorrhages during the first few passages, either after primary isolation or after low-temperature storage, suggests a period of adaptation of the organism to passage.

Although no significant difference was apparent between the intensity of petechiation of different mucosae, some were undoubtedly easier to examine, and easier to distinguish petechiae on, than others. In descending order of ease of examination and distinction, these were vulva-vagina, gums, tongue, and conjunctiva. The appearance of petechiae on a congested background noted by Plowright (1962), was typical of the fatal cases, and it was only such animals that he observed.

The mortality rate of 10% was lower than the 20% estimate of Haig (1966). Laboratory animals are subjected to less stress than those naturally infected, and this might lower the experimental mortality rate. Also a proportion of mild field cases are probably undiagnosed, which would tend to increase the apparent field mortality rate. Alternatively, the strain used may have been mild.

Table 14. Presence and intensity of petechiation at different sites in cattle.

Site	Presence of petechiae		Intensity score (mean \pm s.d.)
	No. showing/Total	%	
Vulva	6/6	100	2.3 \pm 0.8
Conjunctiva	13/15	87	1.6 \pm 0.9
Gums	6/8	75	1.9 \pm 1.2
Tongue	13/15	87	1.9 \pm 1.1

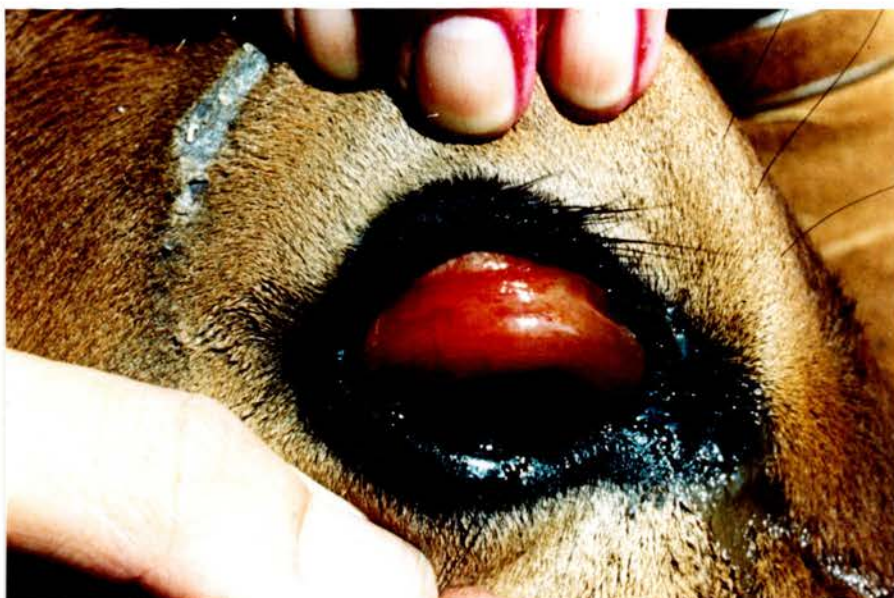


Fig.11 'Poached egg eye' and lachrymation in bovine animal infected with BPF.

CHAPTER 10CLINICAL PARAMETERS

Marked haematological changes have been recorded in BPF infection. In particular these have concerned a leucopenia, but decreases in erythrocytes and thrombocytes have also been noted (Kenya, 1953, 1956; Plowright, 1962; Danskin and Burdin, 1963; Krauss *et al*, 1972).

There are no detailed observations on sequential haematological changes in cattle and sheep experimentally infected with BPF.

A) CATTLEMaterials and Methods

1. Animals and C.ondiri. Eighteen Sahiwal cross cattle infected with N/B strain of BPF were used.

2. Observations. Total leucocyte counts were estimated by electronic counter. Differential leucocyte counts were performed by standard techniques (Dacie and Lewis, 1966). At least 100 neutrophils were counted to estimate the percentage of neutrophil infection, and this figure together with total and differential white cell counts was used to calculate the number of infected neutrophils per cu. mm.

Results

1. Fever. The onset of illness was usually marked by a rise in temperature (Fig. 12, Table 15). The mean incubation period to fever greater than 40.0°C was 5.7 ± 1.9 days, in the range 4-10 days. Fever lasted for 3.1 ± 1.6 days. All the temperature reactions were monophasic.

The reactions had a mean peak of $41.0 \pm 0.6^{\circ}\text{C}$, occurring on day 1.9 ± 0.7 of reaction i.e. usually on the second day.

2. Haematology. Total leucocyte numbers decreased at the start of reaction (Fig. 13, Table 16) from a preinoculation mean of $11,600 \pm 3,400$ per cu. mm., to a mean nadir of $5,300 \pm 2,300$ per cu. mm. on day 2.7 ± 1.6 of reaction. The level returned to normal by day 7.

This leucopenia was made up of falls in the individual white cell types. The eosinophils fell most dramatically, being absent in all except one of the cattle on the first, second, or third days of reaction, and in some cases remaining absent for several days (Fig. 14, Table 17).

Lymphocyte levels started falling by the first day of reaction from a preinoculation mean of $8,900 \pm 2,400$ per cu. mm., to a mean of $3,200 \pm 1,400$ per cu. mm. on day 2.2 ± 1.3 of reaction (Fig. 15, Table 18). Neutrophils dropped during the reaction from $2,800 \pm 1,300$ per cu. mm. before inoculation, to $1,100 \pm 700$ on day 3.7 ± 1.9 (Fig. 16, Table 19). The lymphocyte nadir was significantly earlier than that of the neutrophils ($t = 3.34$, $p < 0.01$). Lymphocyte levels returned to normal by day 7, but neutrophils remained low at this stage.

Monocyte counts did not show a significant rise ($t = 0.78$, $p > 0.05$) or fall ($t = 1.90$, $p > 0.05$) compared with preinoculation levels (table 20).

3. Parasitaemia. The prepatent period to visible parasitaemia coincided with the incubation period to fever in 14 of the 18 cattle. In the other 4 animals, the prepatent period was shorter by from 1-3 days.

Eighty-nine per cent of parasitaemias were monophasic, the

remainder diphasic. Parasitaemias lasted 3.2 ± 1.1 days, similar to the period of fever. The mean peak titre was $10^{2.3 \pm 0.4}$ infected neutrophils per cu. mm., on day 1.9 ± 0.8 of reaction (Fig. 17, Table 21). Mean total parasitaemia was 6.6 ± 2.7 .

B) SHEEP

Materials and Methods

1. Animals and C.ondiri. Thirteen sheep were used, 8 infected with N/O strain, and 5 with a strain from Nderit, near Nakuru.

2. Observations. Observations were made as for cattle.

Results

The results for the sheep reacting to N/O and Nderit strains were calculated separately for each parameter, and then compared by the t test. Of 51 comparisons, 4 were significantly different at the 5% level of probability, and these 4 were all in differing sections of the results. No difference between the two strains having been shown, the results were combined and treated as a single entity.

1. Fever. The onset of illness was marked by a sharp rise in temperature (Fig. 12, Table 15). The mean incubation period to fever greater than 40.5°C was 4.8 ± 1.7 days, in the range 3-7 days. Febrile reaction lasted for 3.4 ± 1.5 days. Eighty-five per cent of reactions were monophasic, the remainder diphasic.

The febrile peak amplitude was $41.8 \pm 0.3^{\circ}\text{C}$, occurring on day 1.9 ± 0.6 i.e. usually on the second day of reaction.

2. Haematology. Leucocyte levels fell at the start of the reaction. Total leucocyte count was depressed to a mean nadir of $2,500 \pm 800$ per cu. mm., occurring on day 3.2 ± 1.3 of reaction (Fig. 13, Table 16). The level returned to normal by day 7. This fall was made up of decreases in the values of the different white cell types.

The eosinophil reaction was most marked, levels falling on the first day of reaction, and being consistently absent on the second day (Fig. 14, Table 17). Levels remained low for at least a week.

Lymphocyte counts decreased by the first day of reaction, a nadir of $1,200 \pm 400$ per cu. mm. being reached on day 2.6 ± 1.1 (Fig. 15, Table 18). Neutrophil levels fell during reaction to 500 ± 300 per cu. mm. on day 4.9 ± 1.8 (Fig. 16, Table 19). The lymphocyte nadir was significantly earlier than that of the neutrophils ($t = 3.58$, $p < 0.01$). Lymphocyte counts returned to normal by day 7, while neutrophil levels rose more slowly.

Monocyte counts varied greatly between individuals, and showed a transitory increase to 380 ± 170 on the 3rd day of reaction (table 20).

3. Parasitaemia. The prepatent period to parasitaemia was in all cases the same as the incubation period to fever.

Sixty-nine per cent of parasitaemias were monophasic, the remainder being diphasic. There was no relation between the sheep with diphasic febrile reactions and those with diphasic parasitaemias.

Parasitaemias lasted 3.7 ± 1.0 days, not including the gaps in diphasic reactions. This was similar to the duration of febrile reaction. The mean peak titre was $10^{2.2 \pm 0.3}$ infected neutrophils per cu. mm., on day 2.2 ± 0.8 of reaction (Fig. 17, Table 21). Mean total parasitaemia was 6.7 ± 1.3 .

Discussion

These results confirm and extend previous reports of marked haematological changes.

The reactions in both cattle and sheep follow a clear sequence: the onsets, peaks, and durations of the febrile and parasitaemia reactions usually coincide; the fall and subsequent rise in lymphocytes occurs earlier than the fall and rise of neutrophils; and eosinophils usually disappear, and stay at very low levels for at least a week.

The BPF reactions of cattle and sheep are compared in Figs. 12 to 17. Although there are quantitative differences in their reactions, the sequential changes are very similar for all the parameters measured. Thus sheep may be considered satisfactory experimental models for BPF in cattle.

Anaemia and thrombocytopoenia develop in BPF infection (Kenya, 1953; Kenya, 1956; Plowright, 1962; Danskin and Burdin, 1963). A series of estimations of thrombocyte and red cell parameters similar to that reported for leucocytes would be valuable.

A comparison between the 2 sheep-adapted strains of different origin showed that they caused very similar reactions. The reactions to all strains are not the same, as a strain not passaged in sheep e.g. N/B strain, causes a much milder or abortive reaction in sheep. Strains may also vary antigenically (Chapter 12).

Table 15. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of febrile reactions ($^{\circ}\text{C}$) in cattle and sheep infected with BPF.

Day of reaction	Cattle			Sheep		
	n	\bar{x}	sd	n	\bar{x}	sd
-1	11	39.2	0.5	12	39.9	0.4
1	18	40.6	0.5	12	41.3	0.5
2	16	40.9	0.7	12	41.6	0.6
3	18	40.4	1.1	12	40.7	0.8
4	15	39.8	0.9	11	40.2	0.6
5	10	39.0	0.8	8	39.9	0.5
6				8	39.7	0.6

Table 16. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of total leucocyte counts ($\times 10^3/\text{cu.mm.}$) in cattle and sheep infected with BPF.

Day of reaction	Cattle			Sheep		
	n	\bar{x}	sd	n	\bar{x}	sd
Before inoculation	15	11.6	3.4			
-2				11	4.6	1.7
-1	9	9.8	4.1	12	4.8	1.2
1	18	6.9	2.5	12	3.8	1.2
2	18	6.6	2.4	12	3.3	1.1
3	18	6.6	2.8	12	3.5	1.1
4	12	6.3	2.9	12	3.4	0.9
5	11	9.1	3.6	8	3.2	1.3
6	8	11.5	4.0	8	4.0	0.8
7	4	12.3	5.0	9	4.6	1.3

Table 17. No. of observations (n), mean (\bar{x}), and standard deviation (sd) of eosinophil counts ($\times 10^2/\text{cu. mm.}$) in cattle and sheep infected with BPF.

Day of reaction	Cattle			Sheep		
	n	\bar{x}	sd	n	\bar{x}	sd
-2	5	7.3	3.8	5	2.5	2.1
-1	8	3.7	3.1	11	3.2	2.9
1	18	1.4	1.3	12	0.5	0.8
2	18	0.7	0.9	13	0	0
3	18	0.2	0.4	13	0.3	0.3
4	10	0.4	0.5	12	0.3	0.3
5	10	1.6	2.4	13	1.2	1.3
6	6	1.1	1.9	8	0.3	0.7
7	7	0.9	1.4	8	0.6	1.3
8				10	0.8	0.9

Table 18. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of lymphocyte counts ($\times 10^3/\text{cu. mm.}$) in cattle and sheep infected with BPF.

Day of reaction	Cattle			Sheep		
	n	\bar{x}	sd	n	\bar{x}	sd
Before inoculation	13	8.9	2.4			
-2				11	2.7	1.2
-1	7	8.1	2.3	12	2.5	1.1
1	18	4.0	1.8	13	1.8	0.7
2	18	4.0	1.8	13	1.4	0.5
3	18	4.5	2.3	12	1.6	0.8
4	11	4.9	2.3	13	2.0	0.7
5	10	6.7	2.8	7	2.0	0.5
6	6	7.6	2.8	8	2.9	0.7
7				10	3.2	1.3

Table 19. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of neutrophil counts ($\times 10^3/\text{cu.mm.}$) in cattle and sheep infected with BPF.

Day of reaction	Cattle			Sheep		
	n	\bar{x}	sd	n	\bar{x}	sd
Before inoculation	14	2.8	1.3			
-2				11	1.5	1.1
-1	9	2.2	0.9	12	1.7	0.8
1	18	2.2	1.1	13	1.8	0.8
2	18	2.1	1.0	13	1.7	0.9
3	16	1.6	0.9	12	1.5	0.6
4	12	1.4	0.7	13	1.0	0.5
5	9	1.7	1.9	7	1.0	0.6
6	7	2.0	1.4	8	0.7	0.7
7				10	0.9	0.8
8				6	1.0	0.3

Table 20. No. of observations (n), mean (\bar{x}), and standard deviation (sd), of monocyte counts ($\times 10^2/\text{cu.mm.}$) in cattle and sheep infected with BPF.

Day of reaction	Cattle			Sheep		
	n	\bar{x}	sd	n	\bar{x}	sd
Before inoculation	14	0.6	0.5			
-2				11	1.1	1.0
-1	8	0.5	0.4	12	2.7	2.2
1	18	0.4	0.2	13	2.3	1.2
2	18	0.3	0.3	13	2.5	1.5
3	17	0.4	0.3	12	3.8	1.7
4	10	0.4	0.3	13	3.3	2.4
5	10	0.6	0.4	7	3.4	2.0
6	6	0.8	0.6	8	3.3	1.2
7				10	3.2	1.5

Table 21. No. of observations, mean (\bar{x}), and standard deviation (sd), of parasitaemia reactions (\log_{10} infected neutrophils/cu.mm.) in cattle and sheep infected with BPF.

Day of reaction	Cattle			Sheep		
	$\frac{\text{no. positive}}{\text{no. examined}}$	\bar{x}	sd	$\frac{\text{no. positive}}{\text{no. examined}}$	\bar{x}	sd
1	18/18	2.0	0.3	13/13	1.8	0.2
2	17/18	2.1	0.7	13/13	2.0	0.7
3	15/18	1.7	0.9	12/12	1.8	0.7
4	5/18	0.5	0.8	9/13	1.1	0.8
5	2/18	0.2	0.6	1/8	0.3	0.7
6	1/18	0.1	0.5			

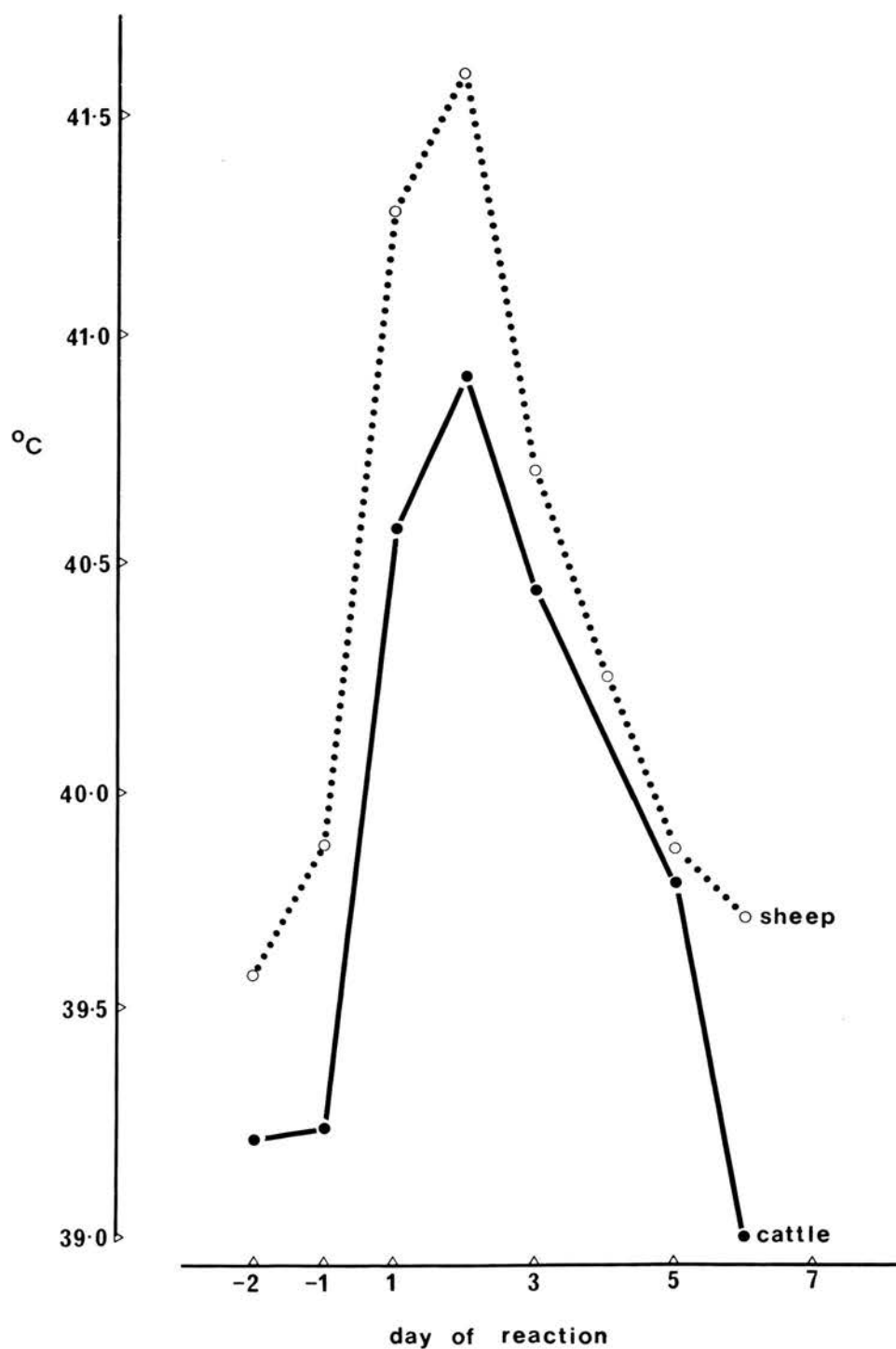


Fig.12 MEAN FEBRILE RESPONSES OF
CATTLE AND SHEEP INFECTED WITH BPF

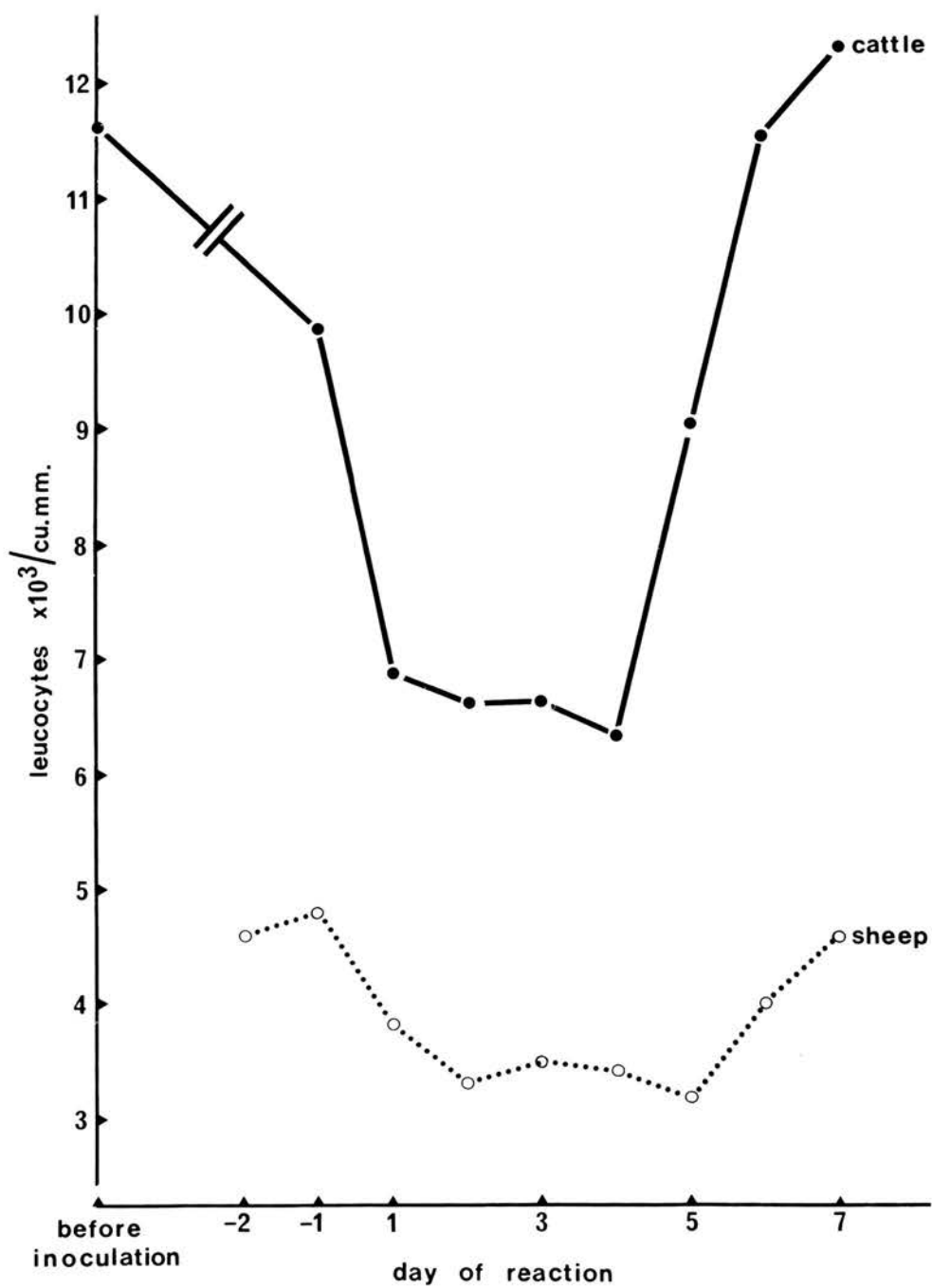


Fig.13 MEAN TOTAL LEUCOCYTE COUNTS IN
CATTLE AND SHEEP INFECTED WITH BPF

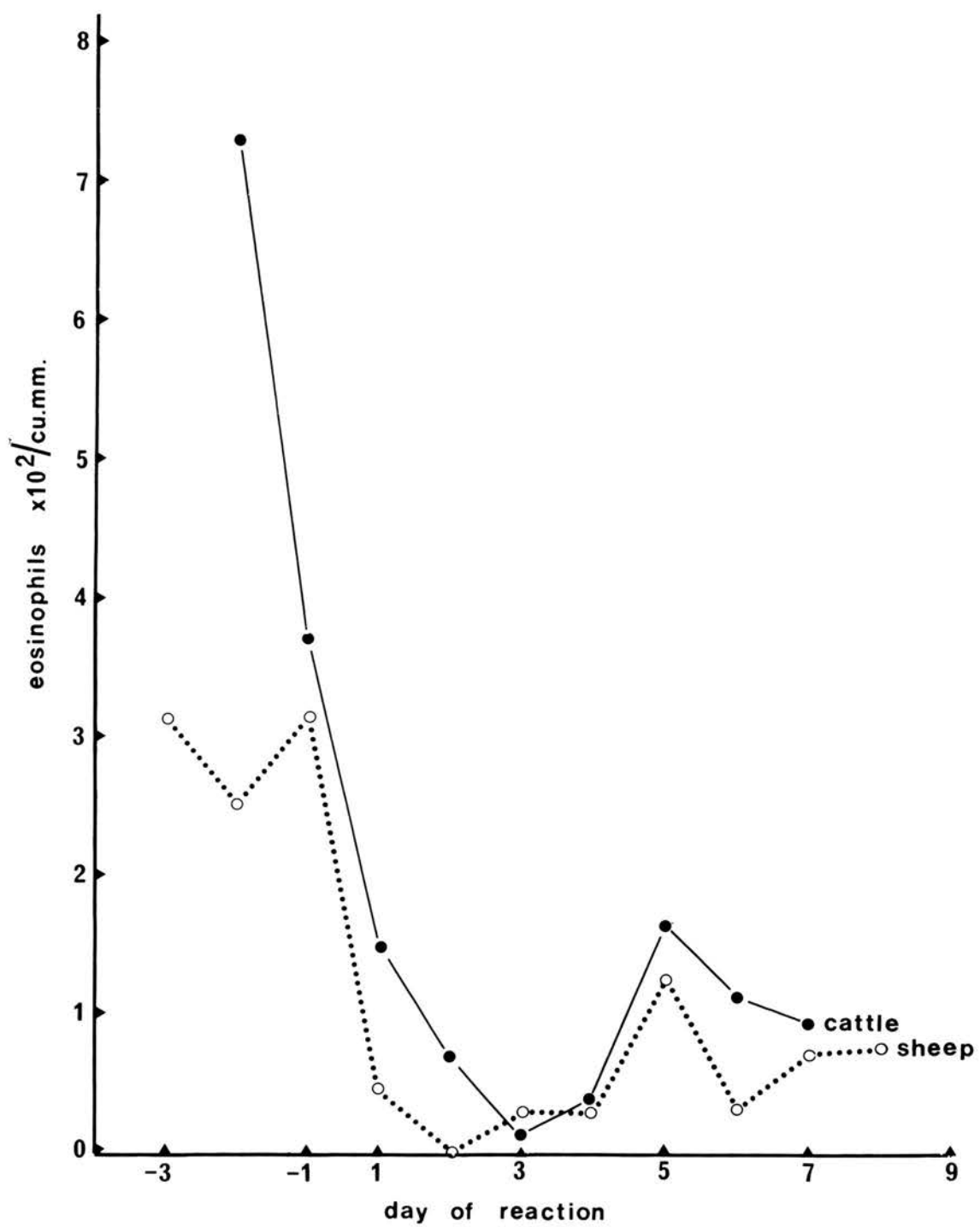


Fig.14 MEAN EOSINOPHIL COUNTS IN CATTLE
AND SHEEP INFECTED WITH BPF

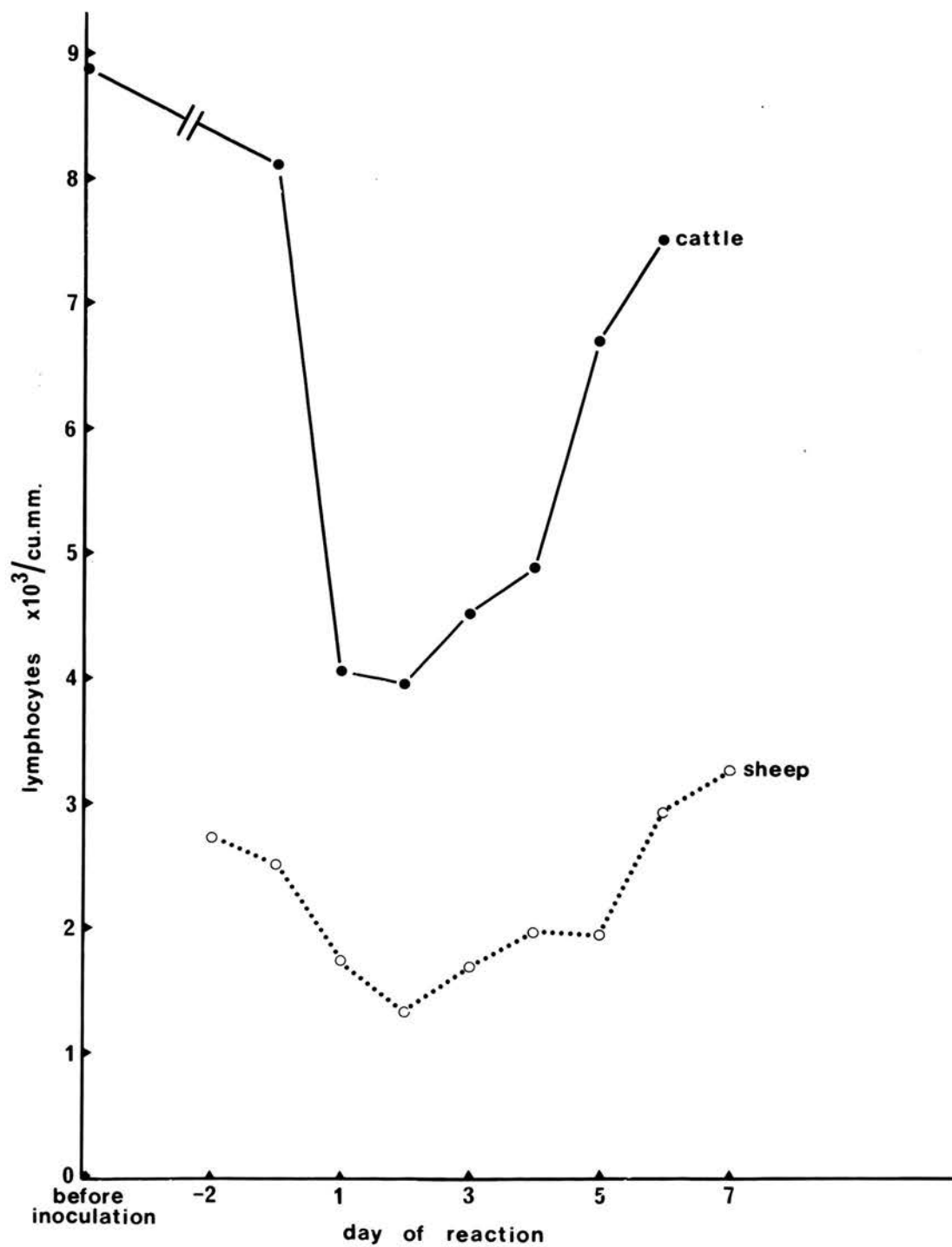


Fig.15 MEAN LYMPHOCYTE COUNTS IN CATTLE
AND SHEEP INFECTED WITH BPF

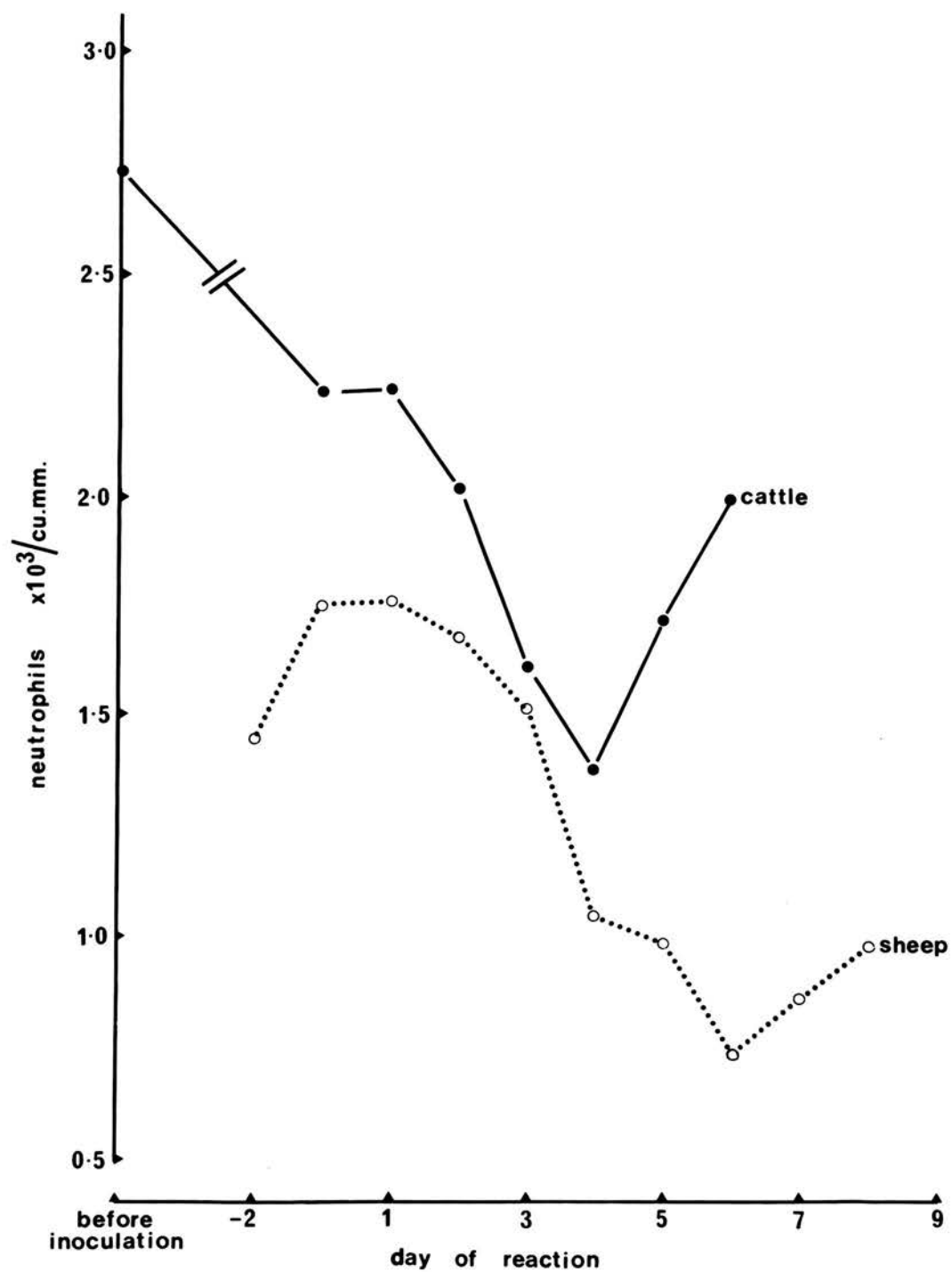


Fig.16 MEAN NEUTROPHIL COUNTS OF CATTLE
AND SHEEP INFECTED WITH BPF

CHAPTER 11LATENT INFECTIONS

Little work has been carried out on latent infections in BPF, although Danskin and Burdin (1963) transmitted BPF from cattle that had recovered for four days from experimental infection.

Both sheep (Foggie, 1951) and cattle (Hudson, 1950) may develop latent infections after TBF. Attempts to confirm this by subinoculating 5 ml of blood from 4 sheep at intervals after recovery were only irregularly successful; this was considered due to the small volumes of blood used. Comparative subinoculations of 1 ml and 100 ml of blood from a recovered sheep were made: only the sheep receiving 100 ml reacted. This confirmed that large volumes were necessary to effectively detect latency.

Materials and Methods

1. Animals and C.ondiri. Cattle and sheep recovered from infection with N/O strain of BPF were used. Eight sheep and 4 cattle served as donors in the subinoculation experiment, and 8 sheep were used for the simultaneous subinoculation and immunosuppression experiment.

2. Subinoculation. 100 mls of blood was collected into heparin (20 units/ml) from each animal at intervals after recovery, and inoculated intravenously to susceptible sheep. At the end of the experimental period, after approximately 6 months, the donor sheep were killed, and 50 ml of a 10% spleen suspension was prepared in phosphate buffered saline (PBS, pH 7.2, containing penicillin and

streptomycin at 200 iu/ml and 200 µg/ml respectively). Ten ml of this suspension was inoculated intravenously, and 40 ml intraperitoneally, to susceptible sheep.

All recipient sheep were monitored after inoculation for evidence of BPF infection.

3. Immunosuppression. Two weeks after recovery from BPF infection, blood was subinoculated from sheep as above. From the same day, a 5-day course of betamethasone¹ at 1 mg/Kg daily was given intramuscularly to the donor sheep. These sheep were subsequently bled daily and blood smears examined, while the recipient sheep were monitored in the usual manner.

Results

1. Subinoculation. Four of the 8 sheep were positive 1 month after primary reaction, two of these still positive after 3 months, but no persistence for longer than 3 months was detected (Table 22). The cattle showed a lower proportion of animals with latent infections, only 2 of 4 animals being positive after 1 week, 1 animal after 1 month, and none thereafter. No isolation was made from any of the spleens.

2. Simultaneous subinoculation and immunosuppression. Only 1 latent infection in the 8 sheep was detected by subinoculation, and none by immunosuppression (Table 23). The betamethasone treatment induced temperature reactions of over 40.5°C in 3 of the sheep, but no parasitaemia was detected. At least 100 neutrophils were counted daily.

1. Betsolan. Glaxo Ltd., England.

Discussion

Latent infections with BPF were shown to occur in a considerable proportion of recovered sheep and cattle. C.ondiri is similar in this respect to other Ehrlichial organisms.

In all, latent infections were detected in 5 of 16 sheep (31%) for 2 weeks or longer.

In some animals, negative results on subinoculation were followed by positive results. It was thought that this was probably not due to the sterility of the blood and subsequent reinfection, but rather to the fact that the level of infectivity in 100 ml blood was insufficient to induce infection on all occasions. Volumes significantly greater than 100 mls would have been desirable but impracticable. However, as shown for TBF, 100 ml volumes are more effective than smaller volumes.

A lower degree of latency was detected in cattle. Whereas the bloods of all sheep were infective 1 week after reaction, only 2 of 4 cattle bloods were infective. Subsequent persistence was detected in one animal only.

Failure to isolate BPF from the spleens is not significant, as the bloods were also noninfective by 6 months after reaction. However, there is other evidence (Chapter 17) that latent infections are more readily detected in blood than spleen.

Immunosuppression was no more effective than subinoculation, and probably less so. The method offers no advantages, is expensive, and laborious in that closer checks have to be kept on the sheep.

It is not known why immunosuppression was not more effective. Large doses of betamethasone were used, and produced a decrease in lymphocytes in all cases. The temperature reactions in 3 of the

sheep were probably due to relapses of intercurrent infections, although they may have been due to low level BPF reactions that were not detected by routine means. A similar method provokes relapses in 50% of sheep recovered from TBF (Scott, G.R., personal communication).

Although considerable latency of BPF infection has been demonstrated, it is not certain that the figures are absolute. In future, it may be possible to develop a more sensitive technique to detect lower levels of infection, which may show more animals with latent infections for longer periods. However, the levels already demonstrated must be considered of importance to the epidemiology of BPF, as recovered animals constitute a continuing reservoir of infection.

Table 22. BPF latency after recovery as detected by subinoculation.

Animal	Time after reaction							Spleen
	1w	2w	1m	2m	3m	4m	5m	
O 166	+	+	+	-	+	-	-	-
O 207	+	-	-	-	-	-	NT	NT
O 235	+	-	-	-	-	-	NT	-
O 240	+	+	+	-	-	-	-	-
O 202	+	-	+	-	+	NT	NT	NT
O 203	+	-	-	-	NT	NT	NT	-
O 249	+	-	-	NT	NT	NT	NT	NT
O 252	+	+	+	-	-	-	NT	-
B 6904	+	-	-	-	NT	NT	NT	NT
B 6905	+	-	+	-	-	NT	NT	NT
B 6910	-	-	-	-	NT	NT	NT	NT
B 6911	-	-	-	-	NT	NT	NT	NT

O sheep

B cattle

+ positive

- negative

NT not tested.

Table 23. Comparison of the efficiency of subinoculation and immunosuppression at detecting latent infections in sheep 2 weeks after BPF reaction.

Sheep No.	Technique	
	Subinoculation	Immunosuppression
206	+	-
91	-	-
219	-	-
220	-	-
3166	-	-
3173	-	-
3176	-	-
3189	-	-

+ positive

- negative

CHAPTER 12IMMUNITY

Resistance to BPF after natural and experimental infection in cattle is known to last for at least $1\frac{1}{2}$ to 2 years, and to be effective between different isolates (Kenya, 1936; Piercy, 1953; Kenya, 1958; Danskin and Burdin, 1963). The only reported work on immunity in experimental sheep found homologous strain immunity declining from 3-4 weeks after infection (Dawe et al, 1970).

The aim of this work was to confirm these results, and investigate homologous and heterologous strain immunity in cattle and sheep.

Materials and Methods

1. Animals and C.ondiri. Fourteen sheep recovered from infection with N/O strain were used. Nine cattle had recovered from BPF; 8 from N/B strain, and one from Mau Narok strain. Challenge inoculations were with N/B, N/O, and Molo strains. Control animals were inoculated at the same time as the challenged animals.

2. Observations. Daily examination of the challenged animals continued throughout the period of reaction of the controls.

Results

1. Sheep. Results are summarised in Table 24.

a) homologous strain challenge. Four sheep that had undergone a primary reaction 6 weeks previously, were challenged with N/O strain. None showed fever or parasitaemia. Two controls reacted normally.

Five sheep that had reacted to BPF 10-12 months previously were challenged with N/O strain. One reacted with both fever and parasitaemia, but the other 4 remained normal.

~~****~~ b) heterologous strain challenge. Five sheep were challenged with Molo strain, 10 weeks after recovery. Four of these reacted with fever and parasitaemia, and the fifth remained normal.

2. Cattle. Results are summarised in Table 24.

a) homologous strain challenge. Four cattle were challenged with N/B strain, 10-12 months after primary reaction. No febrile reaction or parasitaemia was detected in any of these cattle, while 2 controls reacted normally.

b) heterologous strain challenge. Five cattle were challenged with Molo strain, 9-14 months after primary reaction. Four had recovered from N/B strain, and one from Mau Narok strain. Two of the cattle showed low level parasitaemias to a maximum of 1% and 4% for 1 day and 2 days respectively. These reactions were not accompanied by fever or any other sign of clinical illness. The other 3 animals remained normal throughout the experimental period. Two controls reacted typically.

Discussion

The results were in general agreement with those previously published. Cattle were found to have a strong homologous and heterologous strain immunity. The slight breakthrough with heterologous strain challenge was detected only by careful examination of blood smears, and was not important clinically. These findings are

in agreement with those of Kenya (1936), Piercy (1953), Kenya (1958), and Danskin and Burdin (1963).

It is possible that stress of handling may make latent infections of Babesia divergens in cattle become patent (Trees, A.J., personal communication). The very mild reactions in the 2 challenged cattle may have been a result of a similar phenomenon, rather than reaction to challenge.

Homologous strain immunity in sheep was strong, although one sheep reacted when challenged after one year. Heterologous strain immunity in sheep was poor, even as soon as 10 weeks after primary reaction. The reactions of the challenged sheep to Molo strain were mild, but still accompanied by febrile reactions, unlike the heterologous strain breakthroughs in cattle. These results differ from those of Dawe et al (1970), who found poor homologous strain immunity in sheep.

It is apparent that not all BPF strains are identical. This had been noted previously by the author, in that field isolates varied in their ease of adaptation to sheep. There may also be antigenic differences between different strains, as shown by the failure to cross-immunise in sheep and, to a lesser extent, in cattle, although these results must be interpreted cautiously in view of the finding of Dawe et al (1970) of poor homologous immunity. There are grounds for referring to different strains of C.ondiri according to the definition of Dorland (1957).

Table 24. Immunity of sheep and cattle to BPF challenge. The criterion of susceptibility was the presence of a visible parasitaemia.

Species	Challenge	Interval after primary reaction	Number immune	Number susceptible	Immunity %
Sheep	Homologous	6 weeks	4	0	100
		10-12 months	4	1	80
	Heterologous	10 weeks	1	4	20
Cattle	Homologous	10-12 months	4	0	100
	Heterologous	9-14 months	3	2	60

CHAPTER 13DISTRIBUTION OF C.ONDIRI IN TISSUES OF INFECTED ANIMALS

In the past, only limited efforts have been made to transmit BPF with tissues other than blood from infected animals, and results conflict. Unsuccessful attempts to transmit from spleen and liver have been reported (Kenya, 1956), while other attempts succeeded in transmitting BPF with lymph node and spleen suspensions (Kenya, 1960; Danskin and Burdin, 1963; Dawe et al, 1970). Rickettsia-like bodies have been observed in histological sections of heart muscle (Kenya, 1954), in spleen smears (Kenya, 1960; Haig and Danskin, 1962), and in von Kupffer cells in liver (Plowright, 1962).

Materials and Methods

1. Animals and C.ondiri. Blood from an infected sheep was stored in 1 ml amounts in liquid nitrogen, and sheep were infected by the inoculation of the contents of one ampoule. Infected bovine buffy coat cells were similarly stored, and cattle inoculated with the contents of one ampoule. N/O strain was used for both cattle and sheep.

Cattle and sheep at various stages of reaction were the sources of infected tissues after being killed and exsanguinated.

Susceptible sheep were used for titrations.

2. Examination of tissue smears. Impression smears were made of all the tissues sampled, stained Giemsa, and examined for the presence of particles with the morphology of C.ondiri.

3. Preparation of tissue suspensions. Tissues were collected with aseptic precautions, washed free of blood with PBS (containing penicillin and streptomycin at 200 iu/ml and 200 μ g/ml respectively), and cleaned of fat and other extraneous tissue. Where appropriate, they were finely minced, and diluted to 10% w/v or v/v in PBS. A cell suspension was prepared where necessary by disrupting the tissue in a ground glass tissue grinder.

Suspensions of the following tissues were prepared: mesenteric lymph node, mediastinal lymph node, thymus, lung, liver, kidney cortex, spleen pulp, pancreas, cerebrum, vascular endothelium, bone marrow, blood, and plasma.

Blood was collected in EDTA. Plasma was prepared by the centrifugation of blood. Bone marrow was obtained by compressing ribs in a vice, and collecting the expressed bone marrow. Vascular endothelium was stripped from the great veins.

All subsequent tissue dilutions from 10^{-1} were made in PBS with the addition of heparin at 10 units/ml, as clotting was occasionally observed in tissue dilutions.

4. Infectivity titration. All tissues were titrated in sheep on the same day that the donor animal was killed. Each tissue dilution was inoculated to one sheep, by slow intravenous injection. The sheep were then monitored for BPF infection.

Results

In a preliminary experiment, two sheep reacting to BPF were killed, and the tissues listed above with the exception of vascular endothelium were titrated in sheep. The five tissues showing the

highest titre were selected for subsequent routine study. These were lung, spleen, bone marrow, blood, and liver. Plasma was also used initially, but was later omitted after it was found to have a consistently low titre.

1. Sheep. The mean prepatent period after the standard inoculum was 5.1 ± 0.4 days, with the inoculum amount $10^{1.2} \text{ SID}_{50}$. (50% sheep infective doses). Due to the constancy of this prepatent period, it was possible to estimate fairly accurately the stage of prepatency of an animal after inoculation. Those points on the graph (Fig. 18) plotted during the prepatent period indicate this interval as being 5 days.

Sheep were killed at 7 stages of the reaction, from 4 days before expected onset of reaction, to the 17th day of reaction.

a) Tissue titration. The tissue titration results are shown in Fig. 18.

C.ondiri was recovered from the spleen and blood within 24 hours of inoculation. The titres were low, $10^{0.75} \text{ SID}_{50}/\text{g}$ in the spleen, and $10^{0.5} \text{ SID}_{50}/\text{ml}$ in the blood. This indicates a total spleen and blood content of considerably more than the inoculated dose of $10^{1.2} \text{ SID}_{50}$.

Peak titres of $10^{5.5} \text{ SID}_{50}/\text{g}$ in spleen, $10^{4.5} \text{ SID}_{50}/\text{g}$ in lung and liver, $10^{3.5} \text{ SID}_{50}/\text{ml}$ in blood and $10^{3.5} \text{ SID}_{50}/\text{g}$ in bone marrow, were reached during the phase of visible parasitaemia. Titres thereafter declined slowly, being absent from all tissues by day 17.

The growth curves for spleen, lung, and blood were all roughly parallel, the spleen having consistently the highest titre, followed by lung and then blood. Liver and bone marrow curves differed from these, liver titre showing a later peak, and bone marrow having no

clear peak.

At stages of reaction with very low titre, it was not possible to estimate lung titre, as sheep died if amounts equivalent to lg of lung were inoculated.

b) Examination of smears. During the period of visible parasitaemia, BPF bodies were seen in circulating granulocytes and monocytes in blood and occasionally in other tissues. They were sometimes observed in spleen smears, both in mononuclear spleen cells and extracellularly.

2. Cattle. Four cattle inoculated with the standard inoculum of BPF all had prepatent periods of 6 days. The same assumptions of constancy of prepatent period were made as in sheep.

The same 5 tissues were sampled as had been used in sheep. In addition, vascular endothelium was prepared from some animals, but this was discontinued when it was found not to have a high titre.

Cattle were killed at 5 stages of reaction, from 3 days before the expected onset to the 9th day.

a) Tissue titration. The titres in cattle tissues (Fig. 18) are similar to those in sheep tissues. A less accurate picture is available as fewer animals were killed, but spleen again had the highest titre. Peak titres of $10^{5.5} \text{SID}_{50}/\text{g}$ in spleen, $10^{4.5} \text{SID}_{50}/\text{g}$ in lung, liver, and bone marrow, and $10^{3.5} \text{SID}_{50}/\text{ml}$ in blood, were reached on the first day of reaction, and were identical to those in sheep except in bone marrow.

The tissue titres of an animal killed on the 5th day of reaction are not shown in Fig. 18. All tissues in this animal had titres of less than $10^2 \text{SID}_{50}/\text{g}$, which was the lowest dilution used.

b) Examination of smears. During the period when parasites were visible in leucocytes, particles of C.ondiri could be seen in spleen smears, both in mononuclear spleen cells and extracellularly (Fig. 19). During this same period, they were observed on one occasion each in lung smears (Fig. 20) and liver smears (Fig. 21).

Discussion

No system other than sheep or cattle inoculation exists for the assay of C.ondiri. This limits the amount of work that can be carried out. Tissues were taken from only one animal at each stage of reaction, and only one sheep was inoculated with each tissue dilution. In spite of these limitations on accuracy, the pattern shown by these results is consistent enough to support the view that the results themselves are fairly accurate. Most of the curves are similar to population growth curves (Monod, 1949). Some tissues show an eclipse phase, a phase of rapid growth, and a more gradual decline phase.

An exception to this occurred during the decline phase, when a bovine animal killed on the 5th day of reaction was found to have low titres in all tissues. It has been shown elsewhere (Chapter 11) that individual variation occurs after reaction, with some animals developing latent infections and others apparently not. These anomalous results in the decline phase probably represent this phenomenon.

Initial multiplication of C.ondiri appears to take place in the spleen, within 24 hours of inoculation in the case of sheep. Spill-over into other organs, or multiplication in these organs themselves, takes place, and before the onset of clinical reaction high titres are detectable in a range of tissues. Whereas previously it was assumed

that leucocytes were the principal target organ, it is likely that the organism in the leucocytes represents part of this spill-over. The lack of high titre in other tissues is likely to exclude them as important sites of multiplication or pathogenesis.

Visible parasitaemia occurs during the period of highest titre in all tissues. The maximum blood infectivity titres recorded in both sheep and cattle were $10^{3.5} \text{SID}_{50}/\text{ml}$, and these compare with peak titres of visible parasitaemia of $10^{5.2}$ and $10^{5.3}$ infected neutrophils per ml respectively (Chapter 10). This figure for visible parasitaemia is an underestimate, as no account is taken of infected monocytes and eosinophils, although these are relatively few. This suggests that approximately 10^2 infected cells are required to initiate infection in sheep by blood inoculation from both cattle and sheep.

During the peak reaction, C.ondiri is microscopically visible in the blood, and usually the spleen. Where the organism is, and what form it takes in other tissues of high titre and in blood and spleen outwith periods of visibility, are not known. The single observations of the organism in lung and liver of cattle merely demonstrate that such findings were not the norm. It is possible that the organism is present in a submicroscopic form. Electron microscope studies might elucidate the problem.

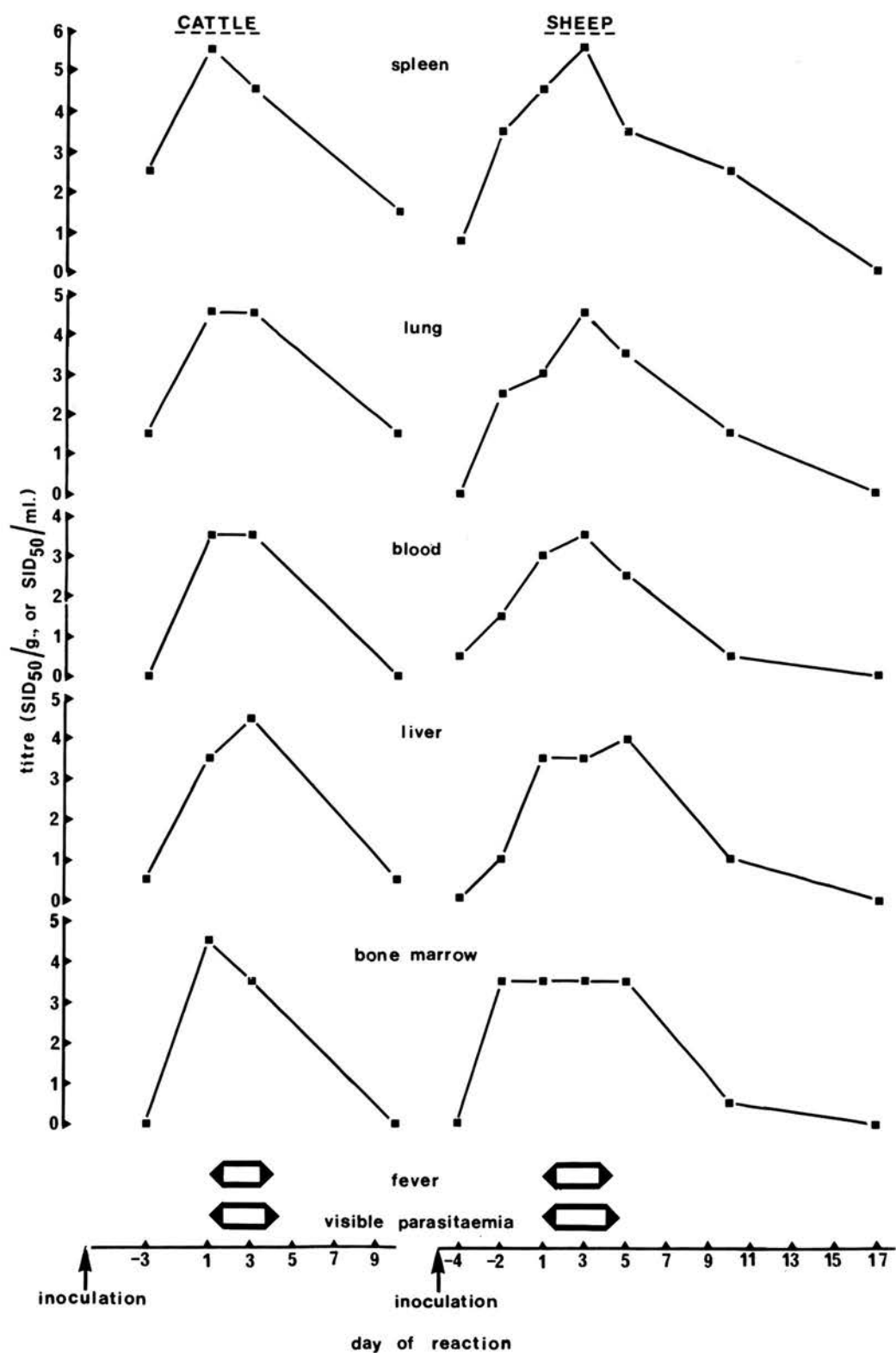


Fig.18 TISSUE INFECTIVITY TITRES IN CATTLE AND SHEEP INFECTED WITH BPF

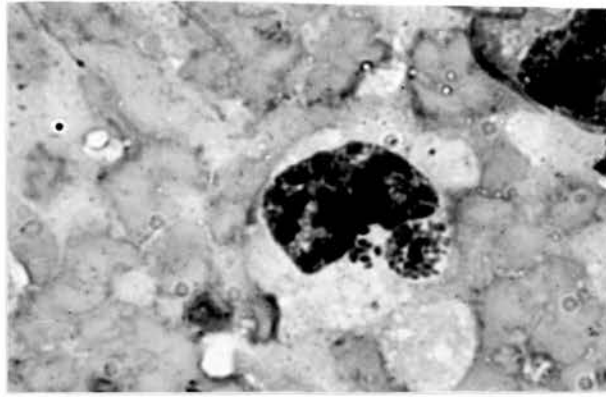


Fig.19 C.ondiri in spleen cell of a bovine animal infected with BPF. X1350.

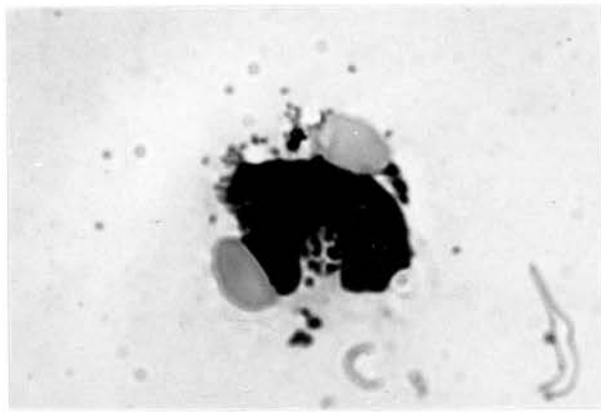


Fig.20 C.ondiri in lung cell of a bovine animal infected with BPF. X1350.



Fig.21 C.ondiri in liver cell of a bovine animal infected with BPF. X1350.

CHAPTER 14SEROLOGICAL STUDIES

No serological test is available for BPF. Attempts at neutralisation in vitro, prophylaxis, or therapy, with convalescent and hyperimmune sera, failed (Kenya, 1956; Kenya, 1957). An effort to demonstrate antigen-antibody reaction between white cell emulsions and sera by the gel precipitation technique did not succeed (Kenya, 1958), and no antibody rise against group-specific chlamydial antigen was shown (Krauss et al, 1972). Specific fluorescence was demonstrated in the indirect fluorescent antibody test (Davies, F.G., personal communication).

Materials and Methods1. Preparation of complement fixation test (CFT) antigen.

Tissues from cattle and sheep reacting to the N/B and N/O strains of BPF respectively were used.

a) Blood. Preparations were made from the blood of 5 sheep and 6 cattle. Blood was collected in heparin at 20 u/ml. Small volumes were collected from the jugular vein of the live animal. To 'bleed out' the animal for larger volumes, cattle and sheep were anaesthetised with xylazine¹ and Nembutal² respectively, and the carotid artery exposed and cannulated.

Whole blood was centrifuged at 1000g, and the plasma removed (Fig. 22). The buffy coat fraction was collected, and the erythrocytes in this fraction lysed with sterile distilled water for 10-60 seconds. Small volumes of hypertonic saline were added to restore isotonicity after the method of Carlson and Kaneko (1973), or large

1. Rompun. Bayer, Germany.

2. Abbott laboratories Ltd.

volumes of isotonic PBS (pH 7.2, no added antibiotics) added to approximately restore isotonicity.

This fraction was then centrifuged again at 1000g to deposit the intact leucocytes and leave in the supernatant fluid the haemoglobin and soluble erythrocyte antigen. The leucocytes were washed twice in PBS, then resuspended in PBS. The leucocyte suspension was disrupted by sonication for three minutes, or by three cycles of alternate freezing and thawing. The disrupted leucocyte suspension was then centrifuged at 1250g for 15 minutes, and the supernatant fluid and pellet collected (Fractions 4 and 5 in Fig. 22). Fraction 4 was the fraction used as TBF CF antigen by Snodgrass and Ramachandran (1971).

To obtain a purer preparation, the leucocyte suspension before disruption was diluted to 5% or less in PBS, and sonicated in an icebath for up to 30 minutes until clear. This preparation was then centrifuged at 40,000g for 30 minutes. The resulting pellet was collected as fraction 7. The supernatant fluid was passed through a Sephadex G-200¹ column, and the eluent fractions collected. These were concentrated by dialysis against Aquacide II², and subsequently against PBS, and are referred to collectively as fraction 6.

On occasion, the erythrolysis was omitted, and the entire buffy coat was disrupted by 3 cycles of freezing and thawing. Centrifugation at 1000g produced a pellet of coarse debris (fraction 1), and the supernatant fluid which was then centrifuged at 6300g. The resulting supernatant fluid and pellet were designated fractions 2 and 3 respectively.

The method used to prepare antigen is summarised in Fig. 22.

-
1. Pharmacia (U.K.) Ltd. London.
 2. Calbiochem, Los Angeles.

The final volume of each antigen fraction was adjusted to approximately 1 ml per 100 mls of original blood.

Merthiolate at 0.01% was added to each preparation.

b) Other tissues. Preparations were made from 9 sheep spleens, 3 cattle spleens, and 3 sheep lungs. The tissues were broken up either by brief homogenisation, or in a ground glass tissue grinder, and were thereafter treated in the same manner as leucocyte preparations.

2. Preparation of immunodiffusion test (IDT) antigen. IDT antigen was prepared from whole buffy coat or spleen leucocytes, which were disrupted by 2 cycles of freezing and thawing. Either the resulting crude suspension was used as antigen, or it was centrifuged to produce fraction 1. Four sheep leucocyte and a sheep spleen preparation were made.

3. Preparation of antigen for the indirect fluorescent antibody test (IFAT). Buffy coat smears from animals with visible BPF parasitaemia were made. They were fixed in acetone at -20°C for 10 minutes, dried and wrapped, and stored at 4°C for up to 2 weeks before use.

4. Sera. Sera were collected serially from experimentally-infected sheep and cattle, and from naturally-infected cattle. 'Hyperimmune' sera were prepared in sheep by 3 inoculations of infective blood, and in rabbits by 2 inoculations 3 weeks apart of antigen preparations with Freund's complete adjuvant. The sheep and rabbits were bled 2 weeks after the last inoculation.

5. CFT. The technique of Grist et al (1966) was followed. Anticomplementary antigen preparations were absorbed overnight at 4°C with heat-inactivated complement.

6. IDT. 1.5% Noble's agar and 4% dextran in veronal buffer was used as gel. After the reagents were placed in the wells, the plates were placed in a humid atmosphere at room temperature, and read after 2 days.

7. IFAT. Test serum was used at a dilution of 1/10 or greater in PBS. The conjugated serum was fluorescein isothiocyanate-conjugated chicken antbovine IgG. Drops of serum were placed on ringed areas of the slide, and incubated in a humidifier at 37°C for 40 minutes. The sera were washed from the slides with PBS, and the slides washed twice for 5 minutes in fresh PBS. Conjugate was added, and the slides incubated and washed as before.

Results.

1. CFT. None of the 5 sheep leucocyte preparations showed CF antigen activity against any sera, but 3 of 6 bovine leucocyte preparations did. Two were derived from fraction 4 and one from fraction 2. Antigen titres were in the range 1/16 - 1/64. Two of the antigens did not produce consistent CF results, but one antigen prepared from fraction 4 gave reproducible results with sera titrations.

This antigen was used with paired preinoculation and convalescent sera from reacting cattle (Table 25). Eight cattle had a mean preinoculation titre of 1/16, which rose after reaction to 1/106. The difference was highly significant ($t = 3.07$, $p < 0.01$).

Four other cattle received similar inocula, but proved refractory to infection (Chapter 8). There was no significant difference between the serum titres before inoculation and after in these cattle ($t = 0.42$, $p > 0.1$).

At 4°C and 20°C, the antigen deteriorated over a few days. At -70°C, the antigen remained stable for a few weeks only.

No specific CF antigen was detected in any preparation from spleens or lungs.

2. IDT. No precipitation lines were produced between antigen preparations and any ovine or bovine sera. Nonspecific lines consistently formed against one rabbit serum.

3. IFAT. All the leucocytes, and particularly the granulocytes, showed bright nonspecific fluorescence. Attempts to reduce this by incorporating 1% Evan's Blue in the conjugated serum were not successful. Fluorescent shapes in the neutrophil cytoplasm with the morphology of C.ondiri could be seen on occasion, but in the presence of the nonspecific fluorescence they were difficult to differentiate from the controls. Reproducibility of results was poor.

Discussion

Specific antibodies to BPF were demonstrated by the CFT and IFAT. Susceptible cattle showed a significant increase in antibody titre, whereas refractory cattle receiving the same inoculum did not. This indicates the specificity of the antigen-antibody reaction.

The technique used successfully was similar to that used by Snodgrass and Ramachandran (1971) to prepare TBF antigen. It is not known why only one of 6 cattle yielded a satisfactory antigen

preparation. The peak visible parasitaemia titre of sheep and cattle reacting to BPF was approximately $10^{5.2}$ infected neutrophils per ml (Chapter 10), which is only 5% of the maximum recorded for TBF (Chapter 2). It is possible that this amount of the organism is not sufficient to produce antigen consistently.

The IFAT was found not suitable due to nonspecific fluorescence. Lohr and Ross (1969) noted the same occurrence when using spleen impression smears infected with Theileria parva. A direct FAT as used by Carter et al (1971) for Ehrlichia canis might have reduced this problem, but would not have been suitable for large numbers of sera.

This work has demonstrated the presence of specific antibodies to BPF. The difficulties of antigen production prevent the application of the techniques used to a routine serological test.

Table 25. Reciprocal CF serum titres of cattle before and after BPF reaction.

Animal No.	Reciprocal CF serum titres	
	Before inoculation	1-3 weeks after reaction
81	8	128
102	8	256
6777	8	64
6773	8	128
6779	8	8
6780	64	128
6621	8	8
6555	16	128

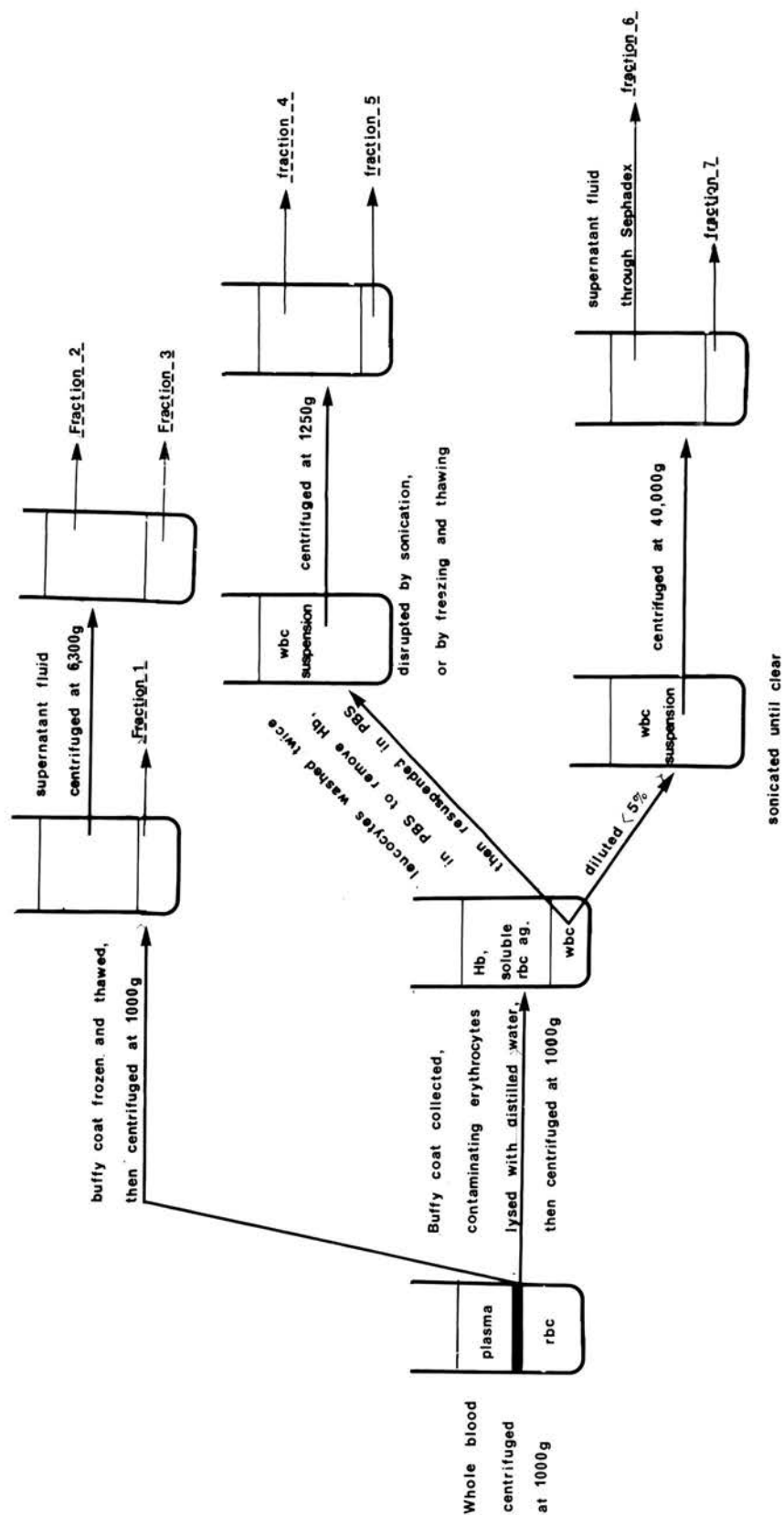


Fig. 22 PREPARATION OF BPf ANTIGEN FROM INFECTED LEUCOCYTES

CHAPTER 15

ATTEMPTED PROPAGATION OF C.ONDIRI IN CELL CULTURE

No method exists for the in vitro cultivation of C.ondiri. Attempts to propagate the organism in calf kidney, sheep kidney or testes monolayers, or a mixed bovine lymphatic and hamster kidney culture, failed (Danskin and Burdin, 1963; Dawe et al, 1970).

This chapter records attempts to apply further tissue culture techniques to the propagation of C.ondiri.

Materials and Methods

1. Alveolar macrophage cultures. Alveolar macrophages cultures from sheep (SAM) and neonatal calves (CAM) were prepared by the methods of Myrvik, Leake, and Fariss (1961), and Appel and Jones (1967). Animals were shot and the trachea clamped before exsanguination. The trachea was removed with the lungs and heart, and taken to the laboratory. The heart was cut away, and the exterior of the lungs washed in PBS (pH 7.5, containing double antibiotic concentrations i.e. penicillin 400 iu/ml, streptomycin 400 μ g/ml). Up to 800 ml warm PBS was poured into the trachea, the trachea clamped, and the lungs gently massaged for a few minutes. The clamp was removed, the lungs inverted, and the fluid collected after filtering through a double layer of gauze. The cell suspension was washed 2-3 times in PBS, and finally the pellet resuspended in growth medium to give a cell concentration of approximately 8×10^5 /ml.

2. Bone marrow culture. Bone marrow cultures from neonatal

calves (CBM) and young sheep (SBM) were prepared by a method similar to that used by Plowright, Parker, and Staple (1968) for pig bone marrow cultures. Ribs were removed from a newly-killed animal, compressed in a vice, and the exuded bone marrow collected. This was filtered, washed once in PBS, and the cells resuspended in growth medium to a concentration of $8 \times 10^6/\text{ml}$.

3. Leucocyte cultures. Blood was collected in heparin at 20 u/ml, centrifuged at 1000g, and the plasma discarded. Distilled water was added to the cells for 30 seconds to lyse the erythrocytes, then isotonicity was restored with hypertonic saline (Carlson and Kaneko, 1973). The resulting leucocyte suspension was washed twice in PBS, and suspended in growth medium to a concentration of $2 \times 10^7/\text{ml}$.

4. Medium. The same growth medium was used for all cultures. It contained Eagle's Minimum Essential Medium¹, with 20% calf serum, 0.044% sodium bicarbonate, penicillin at 200 iu/ml, streptomycin sulphate at 200 $\mu\text{g}/\text{ml}$, and fungizone² or mycostatin² at 5 $\mu\text{g}/\text{ml}$ and 25 u/ml respectively.

5. Incubation. Cells were dispensed in 2 ml amounts to tubes with flying coverslips, or in 15 ml amounts to 100 ml flat bottles. Tubes and bottles were incubated stationarily at 37°C.

6. Infection of cultures. Some cultures were made from tissues of infected animals. To infect those cultures from the tissues of normal animals, the growth medium was discarded, and the cells washed

1. Burroughs Wellcome and Co., London.

2. E.R. Squibb and Sons, Liverpool.

twice with PBS. 0.2 ml of a 1/10 dilution of infected buffy coat cells disrupted by freezing and thawing was added to the tubes, and 2 ml to the bottles. Adsorption proceeded for 2 hours at 37°C, after which the buffy coat was decanted and the cells washed 2-3 times with PBS. Growth medium was added, and the cells replaced in the incubator.

Alveolar macrophage cultures were usually inoculated after 24 hours, and bone marrow cultures after 2-3 days. Leucocyte cultures were from infected animals.

7. Staining. At intervals after infection, coverslips with cells attached were removed, fixed, and stained Giemsa, and examined for the presence of particles with the morphology of C.ondiri.

8. Infectivity. At intervals after infection, the cells were removed from the tubes or bottles with a 0.25% solution of trypsin, and cells and medium inoculated to susceptible sheep. These were then monitored for evidence of BPF infection.

Results

1. Cultures. Cells in all cultures attached readily, and remained healthy for at least 14 days. All consisted of mononuclear cells. Neutrophils did not persist for longer than 3-4 days. Fungal contamination was a common problem with alveolar macrophage cultures, and double-concentration fungizone was used to control this.

2. Infectivity.

a) Alveolar macrophage cultures. CAM cultures inoculated with C.ondiri after 1-2 days were tested for infectivity at intervals

for 2-13 days later. No positive results were obtained. SAM cultures from infected sheep were negative when tested after more than 3 days.

b) Bone marrow. CBM and SBM cultures inoculated with C.ondiri after 2-3 days, were inoculated to sheep from 3-18 days later. No positive results were obtained. A single SBM culture from an infected sheep was found noninfective after 7 days.

c) Leucocytes. Bovine leucocyte cultures from infected animals were found noninfective 4 or more days after initiation.

3. Coverslip examination. No parasites with the morphology of C.ondiri were observed in any cells, and no difference was observed between infected and control cultures.

Discussion

The attempt to propagate C.ondiri in various tissue culture systems failed. Efforts at cultivation of C.ondiri in spleen cultures done concurrently with this work also failed (Sollberg, I., personal communication). Those cultures made from infected tissues had a rapid decrease in titre, and, although the cells remained viable, no infectivity was detectable.

The method used by Nyindo et al (1971) for E.canis culture in leucocytes did not prove suitable. Mononuclear cells alone grew well in leucocyte cultures, and it was these that were parasitised by E.canis.

In contrast, true rickettsiae pathogenic to man can be propagated in several types of primary cell culture and established cell lines (Weiss, 1973).

CHAPTER 16

POSTMORTEM EXAMINATION

Adequate descriptions exist of the lesions caused by fatal BPF (Piercy, 1953; Kenya, 1956; Plowright, 1962; Danskin and Burdin, 1963; and Haig, 1966). The main lesions are widespread submucous and subserosal haemorrhages, with oedema and lymphoid hyperplasia. There are no records of the pathological changes occurring in the mildly-ill majority of sick animals.

This chapter records briefly the fatal natural and experimental cases encountered, and dwells in more detail on the lesions in animals that were killed for experimental purposes.

Materials and Methods

1. Animals and C.ondiri. Eleven Sahiwal crosses and 1 Boran cross were infected with N/B strain. In addition, about 5 natural cases were examined.

2. Observations. The fatal cases were examined as soon after death as possible. Animals killed were examined immediately. The animals were examined for gross lesions only.

Results

1. Fatal cases. Lesions were consistent with those described, of a haemorrhagic syndrome with oedema and lymphoid hyperplasia.

2. Nonfatal cases. A summary of the lesions found in animals

killed at various stages of infection is given in Table 25.

While only 3 cattle showed petechiae on externally-visible mucosae, 8 showed petechiae on at least one site internally. The two most common internal sites were the mucosae of the urinary and gall bladders, with haemorrhages present in 8 and 5 animals respectively. Oedema and lymphoid hyperplasia were noted in only 2 animals each. Excess pericardial fluid occurred in 3 animals.

Discussion

The lesions observed in fatal cases were consistent, and similar to other descriptions.

The lesions found in the majority of those animals which were killed were very slight, but again fairly consistent. Oedema and lymphoid hyperplasia were usually absent, but petechiae were often present, even when absent externally. Submucous petechiae of the urinary bladder were consistently present from the second to the ninth days of reaction.

The presence of petechiae on the urinary and gall bladders during their absence elsewhere has not previously been described. It is not known why these are predilection sites.

Table 26. Postmortem lesions in experimental cattle killed during BPF reaction

ANIMAL	Day of reaction	TISSUE								Other lesions
		External mucosae	Urinary bladder	Gall bladder	Epicardium	Small intestine	Abomasum	Lymph nodes	Spleen	
7429	Incubation period	-	-	-	-	-	-	-	-	
7434	1	-	-	-	-	-	-	-	-	excess pleural, pericardial, and peritoneal fluid
6895	2	-	P	-	-	-	-	-	-	
6840	2	P	P	P	-	-	-	PH	P	abomasal mucosa congested
6696	2	P	P	P	-	-	-	-	P	
6620	2	-	P	-	-	-	-	-	-	
7309	3	-	P	P	P	P	O	H	P	
7309	3	-	P	-	-	-	-	-	-	excess pericardial fluid
7432	5	P	P	PO	-	-	P	O	P	excess pericardial fluid, abomasum congested
7438	9	-	P	P	-	-	-	-	-	

- no lesions detected. P petechiae. O oedema. H hyperplasia.

CHAPTER 17ROLE OF WILD RUMINANTS IN THE EPIDEMIOLOGY OF BPF

Because of the sporadic occurrence and restricted distribution of BPF, it has been speculated that C.ondiri may be harboured by a reservoir host (Piercy, 1953; Haig, 1966). Of domestic ruminants other than cattle, sheep and goats are readily susceptible (Danskin and Burdin, 1963), but there are no published accounts of attempts to experimentally infect species of wild ruminants.

The purpose of this work was to determine the susceptibility of captive wild ruminants to BPF, and, if these were shown susceptible, to attempt the isolation of BPF from wild ruminants in an enzootic area. The work was carried out in cooperation with Dr. L. Karstad, who was concerned mainly with the capture and care of the animals.

Materials and Methods

1. Animals. The following animals were used for experimental inoculation: one impala (Aepyceros melampus), one eland (Taurotragus oryx), one bushbuck (Tragelaphus scriptus), two Thomson's gazelle (Gazella thomsonii), and two wildebeest (Connochaetes taurinus). Adults of either sex were used. All animals were from areas of less than 5000 feet altitude, but the wildebeest were kept in a paddock at 7000 feet altitude, in an area where BPF occurs. With the exception of the wildebeest, animals were housed throughout the experimental period.

Susceptible sheep were used.

2. C.ondiri. The N/O strain was used throughout. Animals were inoculated by the intravenous route with blood from a parasitaemic sheep.

3. Handling. The eland, impala, and wildebeest were anaesthetised by darting from a rifle with an appropriate dose of etorphine hydrochloride¹ and xylazine² (Harthoorn, 1972) before handling. The bushbuck and Thomson's gazelle were caught by hand.

4. Observations. Rectal temperatures were taken, and the animals examined for petechiae and other clinical signs of illness. Blood was withdrawn into EDTA for haematology and infectivity studies.

Blood smears were stained Giemsa, and examined for the presence of C.ondiri. Total white blood cell counts were performed by electronic counter.

5. Infectivity Titrations. The inoculum was titrated in sheep, as was blood taken from the animals before inoculation and at intervals after inoculation. Dilutions were made in PBS, and inoculated by the intravenous route to susceptible sheep. One sheep was used per dilution. The sheep were then monitored for BPF infection.

6. Detection of latency. 100 mls blood was withdrawn into heparin at intervals after recovery, and inoculated into susceptible sheep. These were then monitored for BPF infection.

7. Field isolation. The location chosen for the study was on Ol Magogo veterinary department farm, near Naivasha. This was at an altitude of 6500 feet, and contained an area of thickly-bushed escarpment interspersed with grass. Cattle grazed in this area had a high

1. Immobilon. Reckitt and Colman, Hull.

2. Rompun. Bayer, Germany.

incidence of BPF.

Species were selected for their occurrence at this site and ease of shooting. Kirk's dikdik (Rhynchotragus kirkii), impala, bushbuck, and waterbuck (Kobus ellipsiprymnus) were the species chosen.

The samples collected from the animals were spleen, and up to 100 mls of blood in heparin. These were kept on ice, and transported to the laboratory within 24 hours. 50 mls of an approximately 20% spleen suspension was prepared in a ground glass tissue grinder. 10 mls of this suspension was inoculated to a sheep by slow intravenous injection, and 40 mls by intraperitoneal inoculation. Blood was inoculated intravenously, usually to a different sheep.

To identify an isolate by cross-immunity trials, susceptible sheep and cattle were used, with N/O and N/B as challenge strains.

Results

1. Experimental infection. All animals received large inoculation doses, at least 10^3SID_{50} in each case (Table 27). Blood taken from the animals before inoculation was in all cases negative for BPF.

To determine the blood titres due to persistence of the inoculum, blood was taken from the impala and eland 10 minutes after inoculation, and from the bushbuck 24 hours after inoculation. The impala and eland bloods were non-infective, and the bushbuck blood had a low titre of $10^{0.1} \text{SID}_{50}/\text{ml}$.

To determine if multiplication of C.ondiri had taken place, the animals were bled from 4-7 days after inoculation, some on more than one occasion. The figures in Table 27 represent the highest titres found in each animal during the 4-7 day period. High infectivity titres were detected in the impala, bushbuck, Thomson's gazelle I,

and wildebeest K213. Low levels of infectivity titres were found in Thomson's gazelle II and wildebeest 4, and no infectivity was detected in the eland.

Visible parasitaemia was observed in the impala, the bushbuck, and gazelle I.

Total leucocyte counts varied between the different species. With a definition of leucopenia as a total leucocyte count 20% or more below the starting level, the impala, the bushbuck, gazelle I, and both wildebeest showed a leucopenia.

Temperature reactions of 40.5°C or over were detected in the bushbuck and gazelle I.

Attempts to detect latency were made in only the impala and bushbuck. Latent infection was detected for one month in the impala, which then died of intercurrent disease, and for 2 months in the bushbuck.

The bushbuck appeared dull during febrile reaction. Both gazelle died from 1-2 weeks after inoculation, with haemorrhages, oedema, and lymph node enlargement. No clinical signs were detected in any other animal.

2. Field isolation. Four dikdik, 3 impala, 2 waterbuck, and 5 bushbuck were shot and sampled (Table 28). No isolations were made from dikdik, impala, or waterbuck, but isolations of an organism with the morphology of C.ondiri were made from the bloods of 3 of the 5 bushbuck.

The organism from one isolation was studied in more detail. In sheep and cattle, it caused clinical and haematological changes consistent with those of BPF. One of 2 recovered sheep resisted challenge with the N/O strain; both recovered cattle resisted challenge

with the N/B strain. The organism therefore appeared to be a strain of C.ondiri.

Discussion

Infectivity titres and clinical signs indicate multiplication of C.ondiri in the impala, the bushbuck, gazelle I, and wildebeest K213. There was good correlation between the different signs of multiplication i.e. raised infectivity titre, visible parasitaemia, raised temperature, and leucopenia. The fact that visible parasitaemias were not detected in all cases is not significant, as it has been shown elsewhere (Chapter 13) that visible parasitaemia occurs for a much shorter period in infected animals than does increased infectivity titre. Animals sampled on one occasion only are likely to be within a period of increased infectivity titre, but not necessarily of visible parasitaemia.

The low titres detected in gazelle II and wildebeest 4 were probably a result of slight multiplication of the organism, but persistence of the inoculum cannot be ruled out. The eland appeared resistant to BPF.

Animals other than the gazelle were in a good state of health during the experimental period. The gazelle were newly-captured, and both showed diarrhoea and weakness. This stress may have weakened their resistance to BPF infection, and it is likely that death was due to BPF complicated by the debility.

The absence of clinical disease in infected animals was similar to that found in experimentally-infected sheep and goats.

The fact that isolations were made from 3 of 5 bushbuck indicates either a continuing reinfection cycle, or a much more marked degree of

latency than was detected in sheep and cattle (Chapter 11). The isolations were made at the end of a prolonged dry period, so no exceptional arthropod activity was likely to have occurred.

The susceptibility of several species of wild ruminants to BPF, in addition to the known susceptibility of cattle, sheep, and goats, probably indicates that the majority of ruminants are similarly susceptible, and that latent infections may develop in some of them. Bushbuck in an enzootic area constitute a reservoir of BPF, and other species of wild ruminants may be regarded as potential reservoirs.

Walker et al (1974) found bushbuck to be one of the commonest wild ruminants in most areas where BPF occurs.

Table 27. Reactions of captive wild ruminants to experimental BPF infection.

ANIMAL	INOCULUM (SID ₅₀)	BLOOD TITRES (SID ₅₀ /ml)			VISIBLE PARASITAEMIA	RAISED TEMPERATURE (>40.5°C)	LEUCOPENIA	PERSISTENCE
		BEFORE INOCULATION	AFTER INOCULATION					
			10m - 24h	4-7d				
IMPALA (<i>Aepyceros melampus</i>)	≥ 10 ³	< 10 ⁰	< 10 ⁰	10 ^{2.5}	+	NT	+	1 month
ELAND (<i>Taurotragus oryx</i>)	≥ 10 ⁵	< 10 ⁰	< 10 ⁰	< 10 ⁰	-	-	-	NT
BUSHBUCK (<i>Tragelaphus scriptus</i>)	10 ^{4.5}	< 10 ⁰	10 ^{0.1}	≥ 10 ^{3.6}	+	+	+	2 months
THOMSON'S GAZELLE I (<i>Gazella thomsoni</i>)	≥ 10 ⁴	< 10 ⁰	NT	≥ 10 ³	+	+	+	NT
II	≥ 10 ⁴	< 10 ⁰	NT	10 ^{0.5}	-	-	-	NT
WILDEBEEST (<i>Connochaetes taurinus</i>)	≥ 10 ⁴	< 10 ⁰	NT	10 ^{0.8}	-	NT	+	NT
K213	≥ 10 ⁴	< 10 ⁰	NT	10 ^{2.5}	-	NT	+	NT

NT not tested; + positive; - negative.

Table 28. Isolation of C.ondiri from wild ruminants in an enzootic area.

Species	No. sampled	No. positive
Kirk's dikdik (<i>Rhynchotragus kirkii</i>)	4	0
Impala (<i>Aepyceros melampus</i>)	3	0
Waterbuck (<i>Kobus ellipsiprymnus</i>)	2	0
Bushbuck (<i>Tragelaphus scriptus</i>)	5	3

CHAPTER 18VECTOR STUDIES

Though no prolonged, specific effort was made to identify a **vector**, ticks were collected in enzootic areas from wild animals whenever an opportunity presented itself (chapter 17).

Materials and methods

1. Collection of ticks. Skins from the shot animals were returned to the laboratory in bags, and there searched carefully for ticks. These were removed, and stored at 4°C until identification. Only unengorged ticks were collected.

2. Identification. Ticks were identified by Mr. G. Backhurst.

3. Inoculation. Ticks were ground with sterile sand in PBS (containing added antibiotics). The suspension was centrifuged at 500 rpm, and the supernatant fluid inoculated by the intravenous route to susceptible sheep. These were then monitored for evidence of BPF infection.

Results

The number, species, and infectivity of the ticks collected from different hosts are shown in Table 29. Only the bushbuck carried high numbers of ticks, and all ticks from bushbuck and other hosts were found to be noninfective.

Discussion

Rhipicephalus appendiculatus adult ticks do not transmit Theileria

parva infection to cattle until they have fed for at least 1-2 days (Purnell and Joyner, 1968). The same may be true for any vector of C.ondiri. However, these ticks picked at random from the host would include individuals at all stages of feeding except engorgement, so there should have been considerable numbers at any infective stage.

If one accepts that the restriction on BPF distribution is the presence of a vector, and if the vector is a tick, then the tick concerned is one limited to high-altitude, forest-edge areas. It is one that feeds in its immature stages on wild ruminants which may be reservoirs, and as an adult on cattle. Four ticks have been suggested as fulfilling these theoretical requirements, and these are Rhipicephalus hurti, R.jeanneli, Haemaphysalis aciculifer, and H.parmata (Backhurst, G., and Newsom, R., personal communications).

Three of these four species, H.aciculifer, H. parmata, and R.hurti, were collected in this study, the first two in considerable numbers, but all were found noninfective. Thus the failure to detect infectivity in a large number of these ticks, particularly those from the bushbuck where 60% of the animals were carrying latent BPF, may be a significant negative result.

Table 29. Total number of ticks collected, and their infectivity.

Host	Tick species	No. of adults	No. of nymphs	Infectivity
5 bushbuck	<i>Haemaphysalis aciculifer</i>	185	6	-
	<i>H. parmata</i>	83	0	-
	<i>Rhipicephalus evertsi</i>	3	0	-
	<i>R.hurti</i>	7	0	-
	<i>Hyalomma truncatum</i>	2	0	-
	<i>Ixodes lewisi</i>	3	2	-
4 dikdik	<i>Haemaphysalis aciculifer</i>	3	2	-
	<i>H.parmata</i>	1	0	-
		1	0	NT
	<i>R.evertsi</i>	1	0	-
	<i>R.hurti</i>	1	0	-
2 waterbuck	<i>H.aciculifer</i>	6	0	-
	<i>H.parmata</i>	2	0	-
	<i>R.evertsi</i>	12	0	-
	<i>R.hurti</i>	1	0	-
4 impala	<i>H.aciculifer</i>	4	0	-
		2	2	NT
	<i>H.parmata</i>	1	0	-
	<i>R.evertsi</i>	1	15	-
		1	0	NT

- noninfective

NT not tested.

CHAPTER 19CHEMOTHERAPY

Chemotherapy of BFF has been achieved only by using oxytetracyclines during the incubation period, but not during clinical reaction (Kenya, 1959). There are no quantitative details of the reactions of the treated and control animals.

This chapter records the quantitative effects of treatment of ovine and bovine BFF patent infections with two tetracycline formulations, and a dithiosemicarbazone.

Materials and methods

1. Animals and C.ondiri. Susceptible sheep and cattle were used, infected with the N/O and N/B strains respectively.

2. Treatment. Each animal was treated on the first day of visible parasitaemia. The following experimental groups were used:

a) Pyrrolidino-methyl-tetracycline¹ was inoculated to 8 sheep intramuscularly on 2 successive days, at a rate of 16-24 mg/Kg daily. This tetracycline formulation is very rapidly absorbed, and high blood levels are produced.

b) oxytetracycline hydrochloride² was injected to 8 sheep intramuscularly on 2 successive days, at a rate of 14-20 mg/Kg daily.

c) alphaethoxyethylglyoxal dithiosemicarbazone³ was given in a single intravenous dose to 8 sheep and 4 cattle at a rate of 5 mg/Kg.

1. Reverin. Farbwerke Hoechst AG, Germany.

2. Terramycin. Pfizer.

3. Gloxazone. Burroughs Wellcome and Co., London.

d) Eight sheep and 3 cattle served as untreated controls.

3. Observations. Rectal temperatures were recorded daily after inoculation for all animals, and blood in EDTA was taken from sheep daily after a temperature of 40.0°C was recorded. Cattle were bled into EDTA daily. When C.ondiri was observed in the blood smears of the individual animal, treatment as above commenced. Thereafter the animal was bled daily until 3 negative results were obtained, and temperature recording continued for a further 7 days.

The number of infected neutrophils per cu. mm. was calculated as described previously.

Cattle were examined daily for the presence of petechiae and other clinical signs.

4. Latency. Subinoculations of 100 mls blood were made from some sheep to detect possible latency after treatment.

5. Immunity. Cattle and sheep were challenged after treatment with the homologous strain of BPF.

Results

1. Sheep. The parameters chosen for comparison between the treatment groups were: duration of fever greater than 40.0°C ; duration of visible parasitaemia; and total visible parasitaemia during reaction. The latter two parameters were linked.

a) duration of fever. The temperatures of the treated sheep quickly returned to normal in all treatment groups in a significantly shorter time than the controls did ($p < 0.01$). There were no significant differences between the treatment groups ($p > 0.05$) (Table 30).

b) duration of parasitaemia. On the day following treatment, a few sheep in all groups showed cytoplasmic inclusions in neutrophils that were more rod-shaped than C.ondiri. These were presumed to be degenerating forms of the organism, and were not counted as positive observations. Parasitaemia was not observed on the day after treatment in the 'Gloxazone' group, or in some sheep in the other treatment groups. All treatment groups had a significantly shorter visible parasitaemia reaction than the controls ($p < 0.01$). Between the treatment groups, those treated with 'Gloxazone' had a significantly shorter reaction than those treated with 'Terramycin' ($t = 2.99$, $p < 0.01$).

c) magnitude of parasitaemia. All treatment groups had a significantly smaller parasitaemia reaction than the controls ($p < 0.01$). Between the treatment groups, those treated with 'Gloxazone' had a significantly smaller reaction than those treated with 'Terramycin' ($t = 2.88$, $p < 0.02$).

Blood from 4 sheep treated with 'Gloxazone' was subinoculated 1-2 weeks after treatment to detect possible latent infections. C.ondiri was found to have persisted in one of the 4 sheep.

Subsequent immunity was impaired by all the treatments. Whereas all normal sheep show homologous strain immunity 1 month after reaction (chapter 12), only from 38-75% of sheep in the different treatment groups were found to be immune (Table 30).

No relapses were detected.

2. Cattle. The same parameters were chosen for comparison between the 4 cattle treated with 'Gloxazone' and the 3 controls (Table 31).

Parasitaemias were detected before febrile reaction in all the cattle. After treatment, the temperature rose above 40.0°C in only one of the cattle, while 2 of the controls showed temperature reactions in excess of 41°C. Nevertheless, the differences were not significant ($t = 1.97$, $p > 0.1$).

Parasitaemia reactions were not eliminated as quickly as in sheep treated with 'Gloxazone', but treatment did cause a significant drop both in duration ($t = 3.0$, $p < 0.05$) and magnitude ($t = 4.7$, $p < 0.01$) of parasitaemia compared with the controls.

One control animal developed petechiae on the gums and under-surface of the tongue, and this and another became very depressed. All treated animals remained clinically normal.

The 4 treated animals all withstood homologous strain challenge one month after reaction.

No relapses were detected.

Discussion

All treatments used had a marked effect on BPF reactions, as was expected from their action on diseases caused by related organisms.

With the treatment regimes used, the most efficacious drug was 'Gloxazone', which had a significantly greater effect on parasitaemia than did 'Terramycin'. 'Reverin' was intermediate between the other two drugs.

Very large doses of the two tetracyclines were used, and it is possible that smaller doses, or treatment for one day instead of two, may have proved equally effective.

No relapses were noted, although relapses are common after treatment of TBF with tetracyclines (Evans, 1972).

A shortage of cattle prevented a comparison between the action of these drugs on bovine BPF. However, 'Gloxazone' was shown to significantly decrease the parasitaemia reaction, and all the treated animals remained clinically normal, while two of three controls showed moderate illness.

In most aspects, sheep provide a good model for bovine BPF. It is therefore possible that 'Gloxazone' would provide a better treatment for field cases of BPF than the two tetracyclines, which are widely used at present. A field evaluation would be valuable. Natural cases of BPF are unlikely to be diagnosed on the first day of reaction, and treatment started on the second or subsequent days may be less efficacious. On the other hand, most unexplained febrile reactions are treated with antibiotic before a positive diagnosis is reached.

The susceptibility of some treated sheep to challenge probably indicates that the parasite was destroyed before immune response was fully stimulated. However, all treated cattle were found to be immune.

'Gloxazone' treatment did not completely eliminate C.ondiri from all the sheep, as shown by the latent infection that was detected. The development of a latent infection may be important to the animal's subsequent immunity.

The practice widespread in Kenya of treating BPF by intravenous injection of a turpentine/liquid paraffin mixture must be mentioned here. Laboratory evaluation of this technique would be difficult, as it presumably acts in a haemostatic rather than an antibiotic manner. Danskin and Burdin (1963) found it of no value.

These are the first successful chemotherapy trials on patent BPF infections. The quantitative results provide a standard against which other drugs may be evaluated.

Table 30. Means and standard deviations of reaction parameters of groups of 8 sheep receiving various treatments for BPT.

	Treatment groups			
	'Reverin'	'Terramycin'	'Gloxazone'	Controls
Duration of fever (days over 40°C)	1.13 [±] 0.35	1.38 [±] 0.74	2.00 [±] 1.19	4.00 [±] 1.07
Duration of parasitaemia (days)	1.25 [±] 0.46	1.75 [±] 0.71	1.00 [±] 0.03	4.13 [±] 0.83
Magnitude of parasitaemia	2.63 [±] 1.17	3.51 [±] 1.44	2.00 [±] 0.35	8.89 [±] 2.97
No. immune to challenge	6	3	5	8*

* expected value.

Table 31. Means and standard deviations of reaction parameters of 4 cattle treated with 'Gloxazone' and 3 controls.

	Treated	Controls	P
Duration of fever (days over 40°C)	0.25 [±] 0.50	2.00 [±] 1.73	> 0.1
Duration of parasitaemia (days)	1.75 [±] 0.96	4.00 [±] 1.00	< 0.05
Magnitude of parasitaemia	2.64 [±] 1.43	8.58 [±] 1.97	< 0.01

DISCUSSION

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Comparison of *C.ondiri* and *C.phagocytophila*, and the diseases caused by them.

The close relationship implied by classification of *C.ondiri* and *C.phagocytophila* was amply confirmed.

The 2 organisms are indistinguishable under light microscopy (Figs. 23 and 24), and the small and large forms observed corresponded to those previously described for the organisms.

The close relationship between the organisms extends to the diseases caused by them. Both organisms cause mild disease in sheep. In cattle, TBF is usually mild, but BPF produces a more severe petechial syndrome. However, when severe illness or death does occur in TBF infection, it takes the form of a haemorrhagic syndrome in cattle (Foggie and Allison, 1960; Wilson *et al*, 1964), or a haemorrhagic enteritis of sheep (Foster *et al*, 1968).

The haematological parameters recorded during reactions to BPF and TBF also support the proposition that *C.ondiri* and *C.phagocytophila* are related. The similarity of the sequential haematological changes induced in sheep by the 2 diseases is very marked (Figs. 25-27), although they show differences in magnitude. The mean febrile reactions were also similar (Fig. 28), but the mean of the ovine TBF reaction masks the frequent multiphasic nature of that response.

Visible parasitaemia growth curves (Fig. 29) show the same multiplication rate, but different peaks and durations, and hence different total magnitudes. *C.phagocytophila*-infected neutrophils were 20 times more frequent than *C.ondiri*-infected neutrophils, at the peak titres

on the 2nd day of reaction. On the same day, the number of neutrophils in TBF was twice that in BPF (Fig. 27), but this still implies a C.ondiri cell infection rate at only 10% of that of C.phagocytophila. Duration of parasitaemia in TBF reaction at 6.2 ± 2.2 days (chapter 2) was twice that of ovine BPF reaction at 3.7 ± 1.0 days (chapter 10).

Thus while the general changes in parameters are similar, there appears to be a difference between the two organisms in the magnitude and duration of visible parasitaemia reactions they produce in sheep.

Thrombocyte counts decrease in TBF (Foster and Cameron, 1968b), while thrombocyte and erythrocyte values decrease in BPF in cattle (Danskin and Burdin, 1963). It is not known if these changes occur in ovine BPF, although this is likely. ✓

In other ways, the two diseases also resembled each other. Techniques found appropriate, or often inappropriate, for study of TBF, were found to function similarly for BPF. The major exception to this was the CFT, used successfully for TBF, but with small success for BPF. The most likely explanation for this may be found in the preceeding paragraphs: only 1/20th as many infected leucocytes were present in BPF as in TBF. The mean TBF CF antigen titre was 1/38 (from table 12), which implies an average titre of 1/2 for BPF antigen. This is not sufficient antigen for use in a CFT.

Cryopreservation, as developed by Foggie et al (1966) for TBF, proved suitable not only for C.phagocytophila but also for C.ondiri. This was a significant advance, although Haig and Danskin (1962) had successfully stored portions of infected spleen at -60°C . The long-term preservation of C.ondiri made possible comparison of the immunising

properties of various strains, which would not otherwise have been feasible. It also enabled standard inocula to be given to animals, so that accurate predictions could be made as to the length of the prepatent period. However, the first passage of N/B strain in cattle after storage rarely produced a petechial syndrome, although the reaction otherwise appeared normal (chapter 9). This may have been a coincidental observation, and it has been shown elsewhere (chapter 16) that the absence of petechiae on external mucosae does not necessarily imply lack of petechiation on internal organs. Nevertheless, its occurrence was enough to justify one passage before using the strain for challenge. This may be an aspect requiring further study.

The results for tissue infectivity titrations for both diseases were similar, although techniques differed, and so titres are not comparable. Within 24 hours of inoculation, multiplication occurred in both BPF and TBF, and peak titres coincided approximately with the period of visible parasitaemia. Blood was found to be of lower infectivity than other tissues. The main difference between BPF and TBF was found to be the relative titres of spleen and lung, with spleen in BPF infection showing the earliest and highest titres, and lung fulfilling this role in TBF infection. Both diseases commonly show extracellular organisms in the spleen (Foggie, 1951; chapter 13), and the technique used to prepare the cell suspension from sheep infected with TBF led to loss of any extracellular organisms (chapter 3). Thus, in the writer's opinion, the spleen is probably the major target organ not only in BPF, but also in TBF.

Very little was known previously about tissue distribution of C.ondiri. Unsuccessful attempts to transmit from spleen and liver

have been reported (Kenya, 1956), while other attempts succeeded in transmitting BPF with lymph node and spleen suspensions (Kenya, 1960; Danskin and Durdin, 1963; Dawe et al, 1970). Rickettsia-like bodies have been observed in histological sections of heart muscle (Kenya, 1954), in spleen smears (Kenya, 1960; Waig and Danskin, 1962), and in von Kupffer cells in liver (Plowright, 1962).

No such investigations had been made on TBF. Neither have titrations of tissues from cases of canine ehrlichiosis been made, but isolated facts are available. Spleen (Ewing and Philip, 1966), and lung and cerebrum (Donatien and Lestoquard, 1937), have been shown to be infective. E.canis is more readily observed in lung smears than in blood (Ewing, 1969), and has been observed also in spleen, liver, kidney, and to a lesser extent in other organs (Boal and Sutmoller, 1957; Puxsoll et al, 1972).

du Toit (1970) detected microscopic evidence of multiplication of C.ruminantium in mesenteric lymph nodes before patent reaction. Other tissues were not examined so intensively, although he thought spleen and von Kupffer cells might also be involved. His evidence of multiplication in the mesenteric lymph nodes does not detract from the possibility that the spleen might be the major target organ in heart-water also.

The development of latent infections after apparent recovery is a characteristic not only of BPF and TBF, but also of diseases caused by related organisms. Although both C.ondiri and C.phagocytophila persist in the tissues of recovered animals, the proportion of animals developing this carrier state may be different. By immunosuppressive methods, Dr. G.R. Scott has shown that 50% of sheep recovered from TBF have latent infections when tested from 4-20 weeks later (personal

communication), and Foggie (1951) found that blood from sheep on tick-infested farms was infective at all times of the year. Immunosuppression applied to sheep recovered from BPF failed to find any latent infections; and subinoculation detected 31% carrier animals 2 weeks after reaction, which decreased to none after 4 months. Thus after TBF infection, more sheep develop latent infections, and for longer periods, than after BPF infection.

The strong immunity to BPF in cattle (Danskin and Burdin, 1963) was confirmed in challenges with both homologous and heterologous strains (chapter 12). In sheep, Dawe et al (1970) found weak homologous strain immunity, while the writer found this immunity to be nearly as good as in cattle, but with poor heterologous strain immunity. It is possible that antigenically different strains were present, but too much emphasis cannot be placed on this due to the findings of Dawe et al (1970).

No attempt was made to add to the confusion regarding TBF immunity. However, when a group of 12 sheep kept for serum sampling were challenged 10 months after primary reaction, 11 of these resisted infection completely, and the 12th showed a minimal reaction. These results are perfectly unambiguous indicators of good homologous strain immunity, but no doubt other workers results seemed equally clear.

Hudson (1950) in cattle and Foggie (1951) in sheep observed a phenomenon of susceptibility to TBF challenge for a few weeks after reaction, which then developed to immunity. After several months, the animals were again found susceptible. This was suggested by Hudson (1950) as being due to an unstable premune state during the period immediately after reaction, which then gave way to a period of sterile and complete immunity, which subsequently waned. However,

Dr. G.R. Scott (personal communication) found latency in 50% of animals up to 20 weeks after reaction, and no correlation between latency and immunity.

Tuomi (1967b) speculated that the primary defence of animals against TEF might not be specific immune mechanisms, but could be through nonspecific mechanisms.

Since studies on BPF and TEF immunity involve very simple experimental procedures, the differing results obtained by various workers present something of an enigma.

Neither C.ondiri nor C.phagocytophila has been propagated in any in vitro system. Different methods were used by the writer in cell culture attempts for both organisms, but with uniform lack of success. Other workers (Danskin and Burdin, 1963; Dawe et al, 1970; Davies, F.G., personal communication; Hudson, 1950; Foggie, 1951; Tuomi, 1967a; and Thrushfield, M., personal communication) have used embryonated eggs and a variety of primary and continuous cell culture systems; none have been successful.

Consideration of the culture methods for related organisms does not prove helpful. Nyindo et al (1971) propagated E.canis in leucocyte cultures from infected dogs; these were mononuclear in character, and were not found appropriate to C.phagocytophila by the author, or to C.ondiri by Dr. Ø. Ødegaard (personal communication). No application has yet been made of this method for study of E.canis.

No method exists for culture of any other of this group of organisms. The cultural characteristics of members of the genus Rickettsia are so different, in that they may be cultivated with relative ease in arthropod tissues, embryonated eggs, and cell culture (Weiss, 1973), that similar methods are obviously not applicable.

Nevertheless, the greatest stimulus to study of the Cytoecetes could come from development of an appropriate technique for in vitro culture.

The small laboratory rodents have been extensively tested for BPF and TBF susceptibility. Attempts to infect splenectomised or immunosuppressed mice, guineapigs, and hamsters, with C.ondiri have failed (Cooper, 1973). Foggie and Hood (1961) adapted strains of C.phagocytophila to normal and splenectomised guineapigs, and splenectomised mice, but the infection remained mild and irregular. The unsuitability of laboratory mice has been shown by Foggie (1951) and in this study (chapter 6), due to the possible latent presence of other organisms with similar morphology. This is reinforced by Tyzzer (1938), who isolated C.microti from voles, and transmitted it to the white rat and several mouse species.

C.ondiri and C.phagocytophila react similarly to chemotherapeutic agents. Evans (1972) working with TBF in sheep, found chloramphenicol inactive, penicillin and streptomycin combination slightly active, and dithiosemicarbazone, oxytetracycline, and sulphamezathine to be active in that order. Detailed results were not available for BPF treatment, but Haig and Danskin (1962) found oxytetracycline effective during the incubation period, but not during clinical reaction. The writer (chapter 19), with ovine BPF, found that dithiosemicarbazone was more active than two tetracycline formulations in treating patent infections, and that the dithiosemicarbazone was also active against bovine BPF.

The same agents, dithiosemicarbazone and tetracycline, are used to treat canine ehrlichiosis, both prophylactically and therapeutically (Walker et al, 1970; Seamer and Snape, 1972).

It is probable that all the ehrlichial organisms are similar in their sensitivity to antibiotics.

In short, the differences between C.ondiri and C.phagocytophila are mainly quantitative, not qualitative. Animals infected with C.phagocytophila have a reaction of greater magnitude, a greater degree of latent infections, and possibly a lesser degree of immunity, than do animals infected with C.ondiri. One difference between them is the clinical petechial syndrome produced in cattle by C.ondiri, but even this may occasionally be present in C.phagocytophila infection. Another possible difference is that TEF is tick-borne, while the vector of LPF is unknown.

In areas of Scotland where TEF is endemic, sheep and cattle appear adapted to C.phagocytophila, while in Kenya, they do not appear well adapted to C.ondiri, with cattle less so than sheep, although it is possible that native Zebu cattle are well-adapted. Lapage (1958) stated that some hosts and parasites undergo a process of mutual adaptation, to the benefit of both. This tends towards a state of commensalism, with the minimum of inconvenience to the host compatible with sufficient benefit to the parasite.

It is possible that C.ondiri and C.phagocytophila are strains of the one organism, at different stages of this process of adaptation. C.ondiri causes reactions lower in magnitude but more severe in effect than C.phagocytophila, and is not such an efficient parasite in that it is more likely to be eliminated from the host, leaving immunity that will prevent reinfection. Cattle may be an aberrant host in this respect, with C.ondiri being much better adapted to other species such as wild ruminants.

The precise relationship will remain a matter for conjecture

until serological or crossimmunity tests are carried out. At the least, TBF in sheep was a useful model for the study of BPF in cattle.

Other Cytoecetes and Ehrlichia organisms may be similarly very closely related. The organism of equine ehrlichiosis is transmissible to dogs (Gribble, 1969), and so may be the same as that Cytoecetes found in dogs. Similarly, E.bovis is transmissible to sheep (Donatien and Lestoquard, 1937) and pigs (Rioche and Bourdin, 1968); it occurs in the same area geographically as T.ovina and R.suis; and so it is possible that T.bovis, T.ovina, and R.suis are the one species.

Pathogenesis

The pathogenesis of BPF and TBF is not understood. Haematological changes commence before onset of visible parasitaemia, with a fall in lymphocytes and eosinophils. Several factors may contribute to the lymphopenia: there may be interference with lymphocyte production by the organism known to be multiplying in the spleen; the organism may have a toxic effect on lymphocytes, causing their destruction, as may be the case in some viral infections (Theelock and Toy, 1973); or it may be due to a subclinical stress associated with multiplication of the organism, which can lead to a decrease in lymphocytes and the disappearance of eosinophils from the circulation (Schalm, 1965).

Taylor et al (1941) and Foster and Cameron (1968b) associated the neutropenia of TBF with aplasia of the bone marrow. This may be the case in BPF, and it may explain also the thrombocytopenia seen in both diseases. However, in view of the fact that neutropenia occurs just after peak parasitaemia, it is likely that some physical destruction of the parasitised neutrophils also occurs, as suggested by Tuomi and von Bonsdorff (1966).

The neutropoenia of TBF infection has been associated with exacerbation of a staphylococcal infection of lambs (Foggie, 1956; 1957), although Foster and Cameron (1968a) found that some cases of natural staphylococcal pyaemia occurred before the neutropoenia of TBF. The lymphopoenia in TBF infection is equally marked, and occurs before the neutropoenia (chapter 2). With modern knowledge of the importance of lymphocytes to immunity, the fall in lymphocytes is clearly more important in facilitating staphylococcal pyaemia than the decrease in neutrophils.

The causes of the haemorrhagic syndrome of BPF in cattle are not known. The thrombocytopoenia may be involved, interfering with the normal haemostatic role of the platelet. Increased vascular fragility may also occur in thrombocytopoenia (Castaldi, 1968). However, thrombocytopoenia occurs in TLF (Foster and Cameron, 1968b), and probably in ovine BPF, without a haemorrhagic syndrome, so it is probable that other factors are also involved.

Members of the genus Rickettsia multiply in the capillary endothelial cells, thus damaging the endothelium (Snyder, 1965). The agent of equine ehrlichiosis causes vasculitis, and localised petechiae and oedema (Gribble, 1969). C.ruminantium is also found in vascular endothelial cells, where its presence leads to distension but no other apparent damage (Haig, 1955). E.canis has been observed in endothelial cells of lungs of dogs in early experimental infection (Hildebrandt et al, 1973). C.ondiri has not been demonstrated visually in endothelial cells, and it was shown in this study that the intima of large veins from animals with BPF was less infective than some other tissues (chapter 13). Thus although the organism is probably present in endothelial cells, its limited presence is probably not sufficient to account for the haemorrhages.

The other possible cause of the petechiae is the occurrence of a toxin in BPF infection. Members of the genus Rickettsia possess endotoxins, which may cause haemolysis and increased vascular permeability (Clarke and Fox, 1948). C.ruminantium may liberate a toxin responsible for increased permeability of the capillary walls (Neitz, 1966). No toxic effects in BPF have been demonstrated by the writer by inoculation of infected leucocytes or plasma to laboratory rodents.

Of the three factors mentioned i.e. thrombocytopoenia, endothelial damage, and toxin production, it is not known which if any is responsible for the petechial syndrome in BPF.

In dogs with tropical canine pancytopenia, there is hypergamma-globulinaemia (Burghen, Leisel, alker, Nims, Huxsoll, and Hildebrandt, 1971), and perivascular plasma cell infiltration (Nims et al, 1971). These findings may indicate that hypersensitivity or autoimmune reactions play a part in the pathogenesis of TCP. However, there is no perivascular cell infiltration in BPF or TEF, so it is unlikely that the same mechanisms are operative.

C.ondiri and C.phagocytophila may be present in a submicroscopic form during the incubation period, and during patent reaction in tissues other than leucocytes and spleen. This may be the case also with C.ruminantium, which is present but not visible in circulating blood (Haig, 1955).

In latent infections of BPF and TEF, the organism may be detected by subinoculation of blood. No studies have been carried out to find the principal site of persistence, if one other than the blood exists. The spleen is the obvious site, as in late BPF infection

it still showed the highest infectivity titre (chapter 13). However, it must also be present in other tissues, as splenectomy may provoke TBF relapse (Foggie, 1951). Moreover, isolations from bushbuck with what were presumed to be latent infections were always made from the blood, and the spleen from these animals was always negative, although up to 10g was used (chapter 17).

B.canis persists in endothelial cells in higher titre than in blood (Donation and Lestoquard, 1937), and R.prowazekii may also persist in endothelial cells (Jadin, Creemers, Jadin, and Giroud, 1968). It is possible that endothelial cells may prove to be the site of persistence of C.ondiri and C.phagocytophila also, despite the fact that C.ondiri is not present in endothelium in high titre during clinical reaction.

Diagnosis

Diagnosis of BPF is not difficult if fresh samples are obtained. Observation of the organism in blood or spleen smears is usually possible in these cases. Otherwise, blood, lung, or spleen suspensions may be inoculated to susceptible sheep. From this work, it appears that spleen is the tissue of choice. Sheep will normally react to field strains, although in one case a blind passage was needed to produce patent infection.

However, field conditions in Kenya are not ideal, and for reasons of distance or environment good samples are not readily obtained. Recognition of the characteristic morphology of C.ondiri is not possible if samples are not taken immediately after death, and infectivity also drops quickly if tissues are not stored at a cool temperature. There are many instances of a haemorrhagic syndrome similar to BPF occurring to the north of the presently-recognised area i.e.

further from the laboratory, in Uasin Gishu and Trans Nzoia. It has not been possible to diagnose PPF in these cases, perhaps because of the distances involved.

Although the disease has been diagnosed only from the highlands of Kenya, it would be surprising if it did not exist in geographically similar areas in neighbouring countries. There are reports of a clinically similar disease in areas of northern Tanzania (F.G. Davies, personal communication; D. Le Riche, personal communication).

In the course of this work, the area where PPF is known to occur was extended to Olo in the northwest, Subukia in the northeast, and Mau Narok in the west, by means of positive isolations. A serological test is necessary to define the range completely.

Conclusions

The rickettsia-like organisms occurring in the neutrophils, tentatively classified as Cytoecetes, are a fairly homogeneous group. Indeed, although five possible members have been described, it may be that there are only three valid species, as C.ondiri and C.phagocytophila may be one species, and equine and canine Cytoecetes may be one species. The host range of C.microti seems sufficiently different from the others to leave it as a separate entity.

Whether Cytoecetes and Ehrlichia are validly classed as separate genera is not clear. Each undoubtedly contains a group of organisms with a single clear difference between the members, that of the principal leucocyte in which they are visible. However, there is overlap even in this aspect, as Cytoecetes are sometimes observed in monocytes, and Ehrlichia occasionally in neutrophils. One other possible difference is the ease with which Ehrlichia spp. are observable in a variety of tissues other than blood, while Cytoecetes spp.

are commonly seen only in blood and spleen. Otherwise, the organisms have much in common, as is clear from the introduction to this thesis.

The organisms causing BPF and TBF are difficult to study with virological techniques, although they appear to be obligatory intracellular parasites. Conventional methods of cell culture, and most serological methods, do not seem appropriate. C.ondiri and C.phagocytophila can be assayed only in sheep. What can be investigated, however, is the course of the diseases in infected animals, and it is hoped that these studies have contributed to knowledge in this respect, in addition to having proved the feasibility of a serological test for at least one of these organisms.

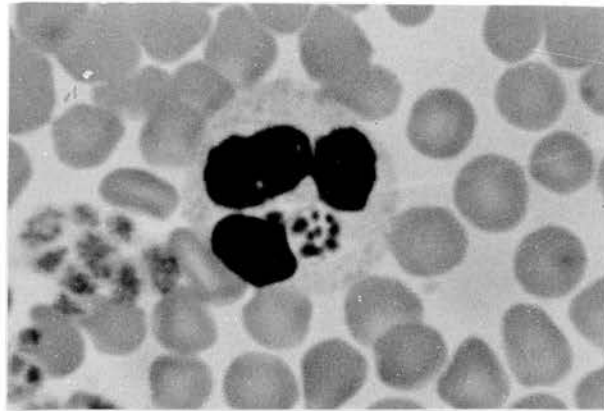


Fig.23 C.phagocytophila in a neutrophil of a sheep. X1800.



Fig.24 C.ondiri in a neutrophil of a sheep. X1800.

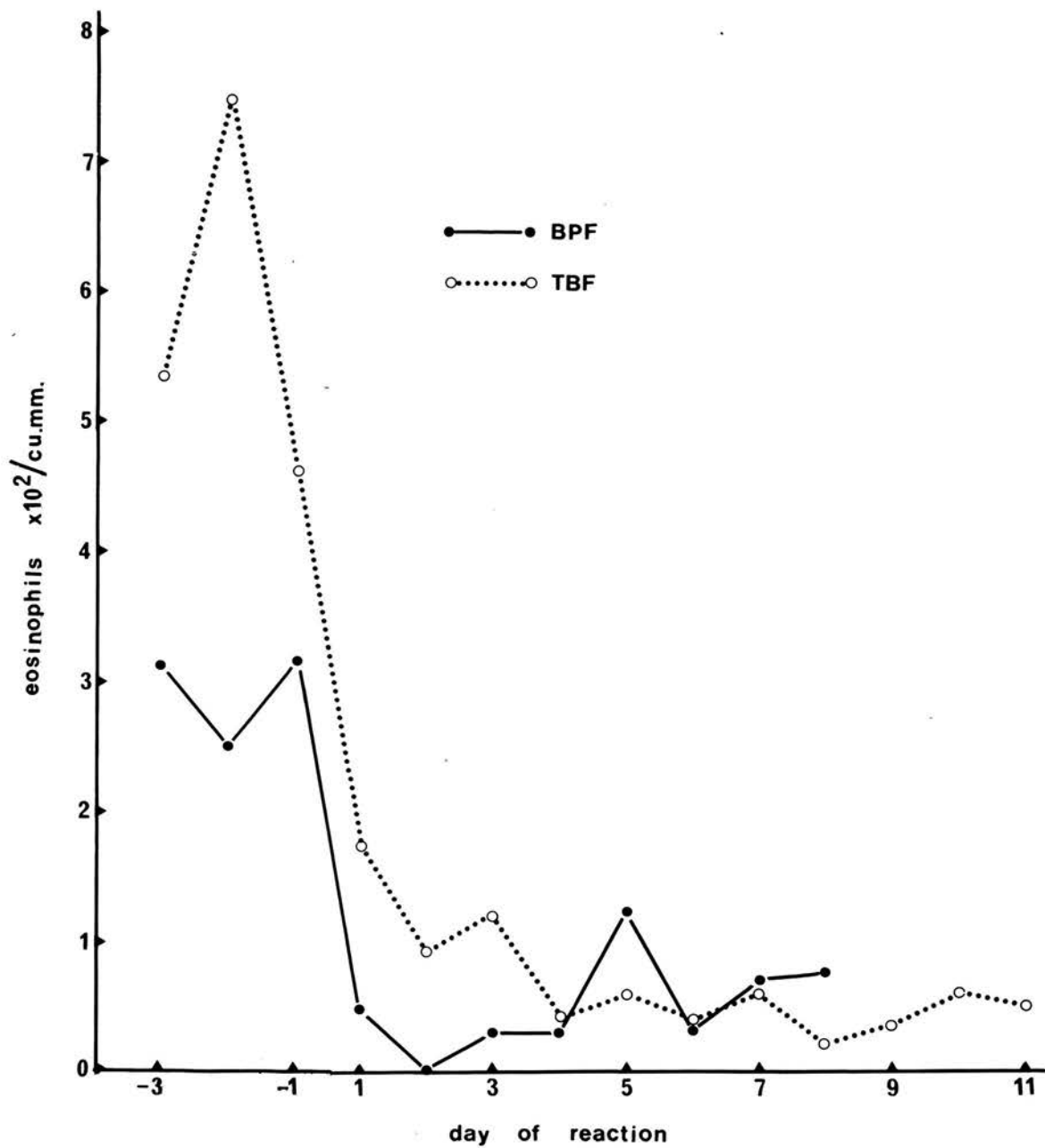


Fig. 25 MEAN EOSINOPHIL COUNTS IN SHEEP

INFECTED WITH BPF AND TBF

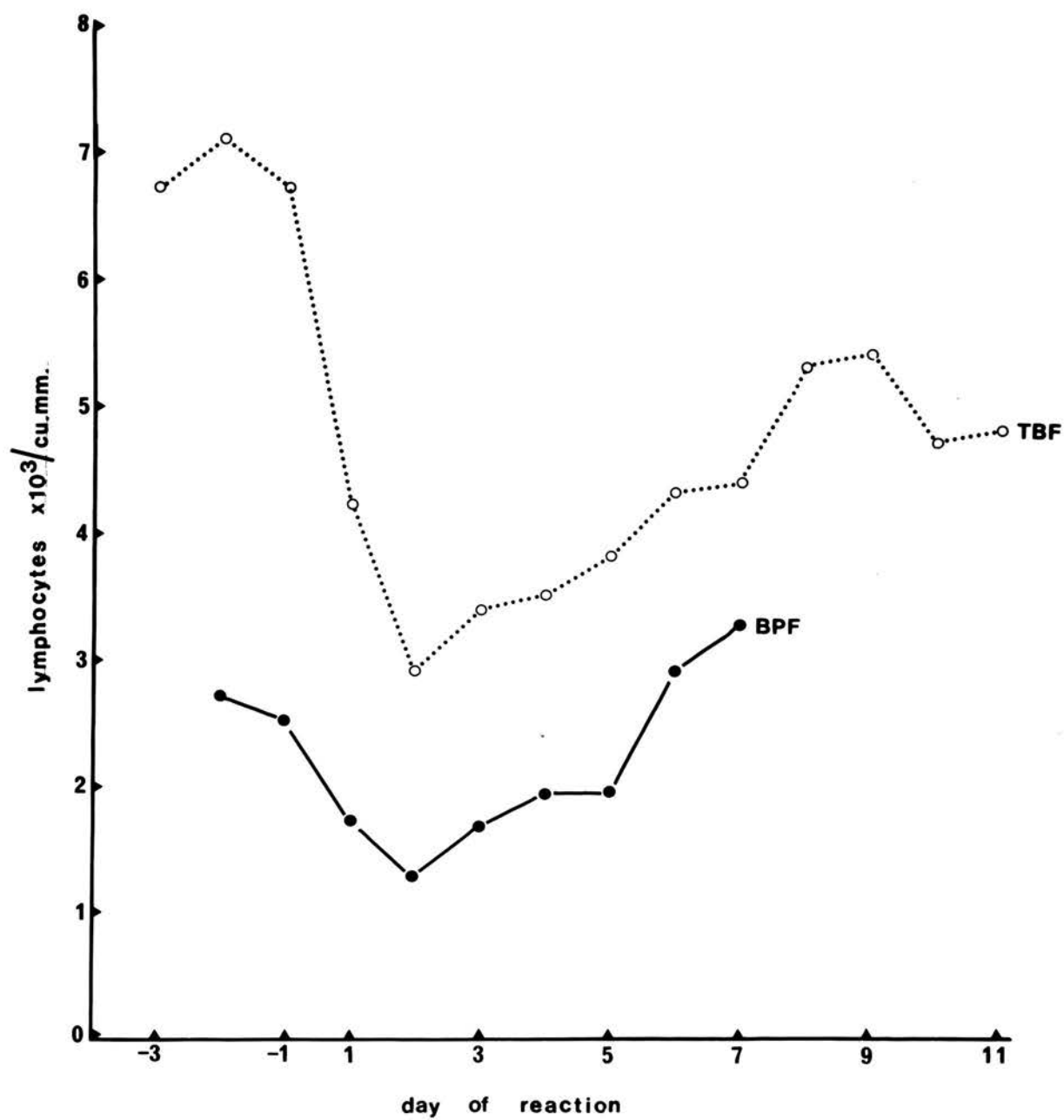


Fig. 26 MEAN LYMPHOCYTE COUNTS IN SHEEP
INFECTED WITH TBF AND BPF

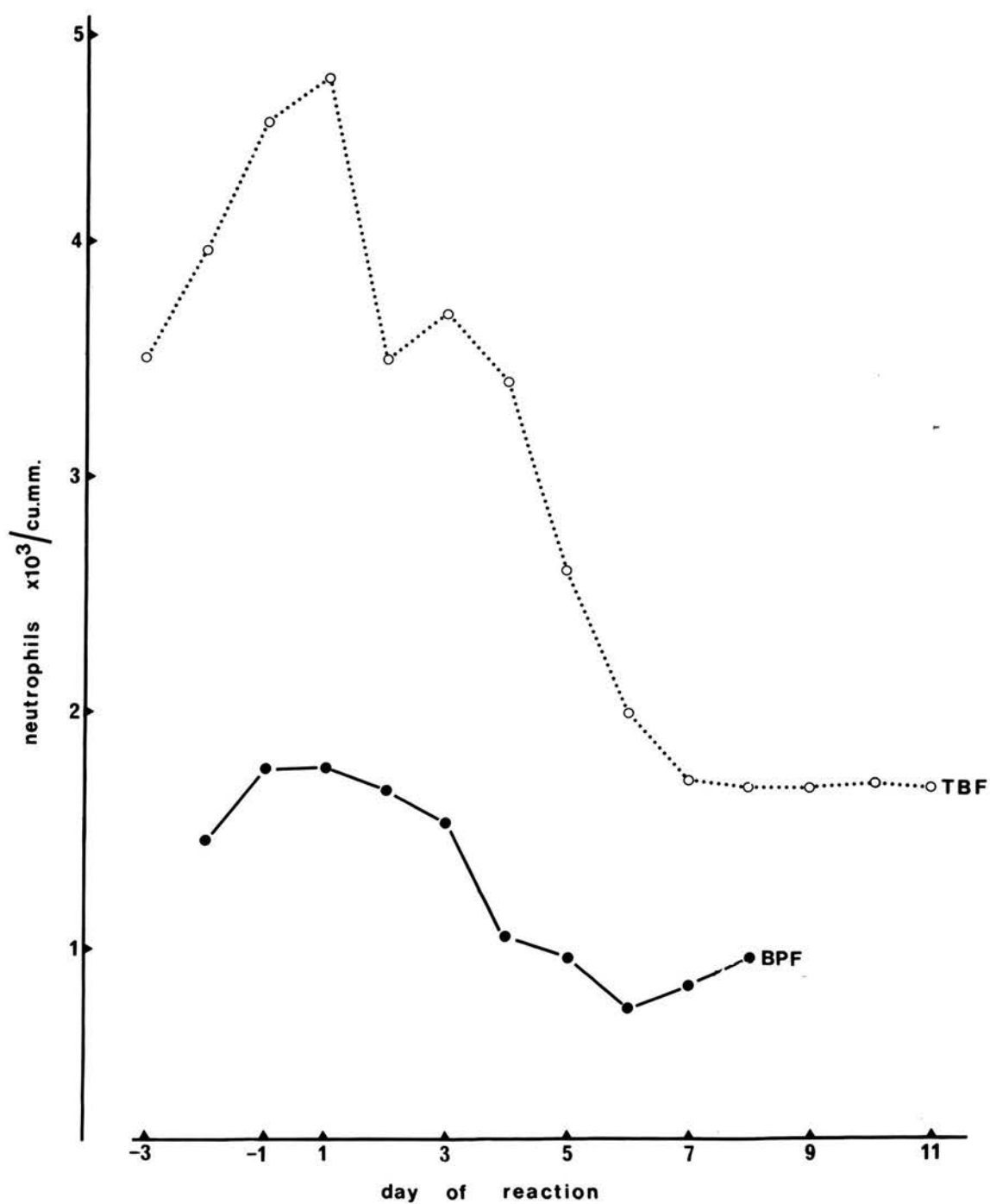


Fig.27 MEAN NEUTROPHIL COUNTS IN SHEEP

INFECTED WITH BPF AND TBF

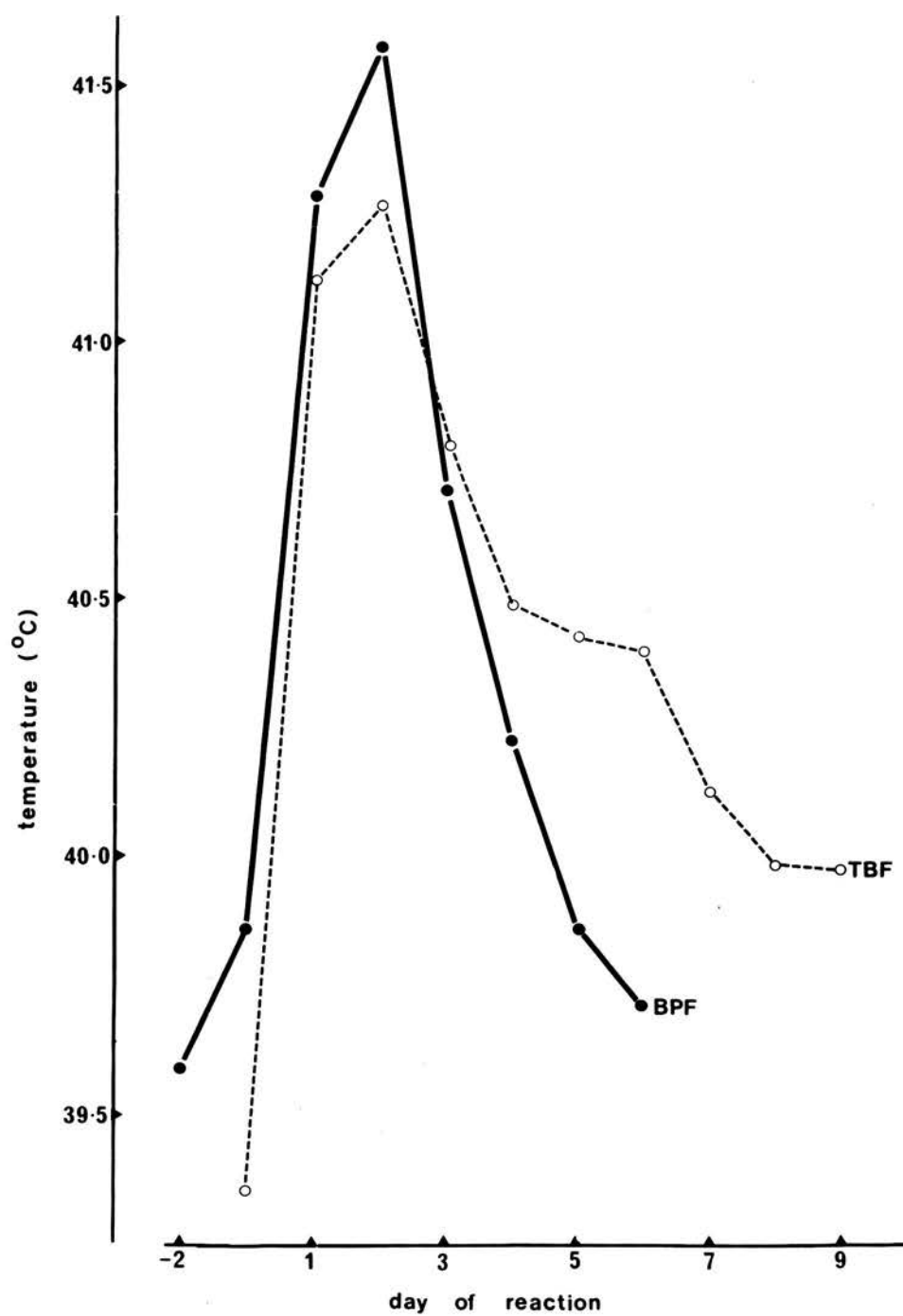


Fig. 28 MEAN FEBRILE REACTIONS IN SHEEP
INFECTED WITH TBF AND BPF

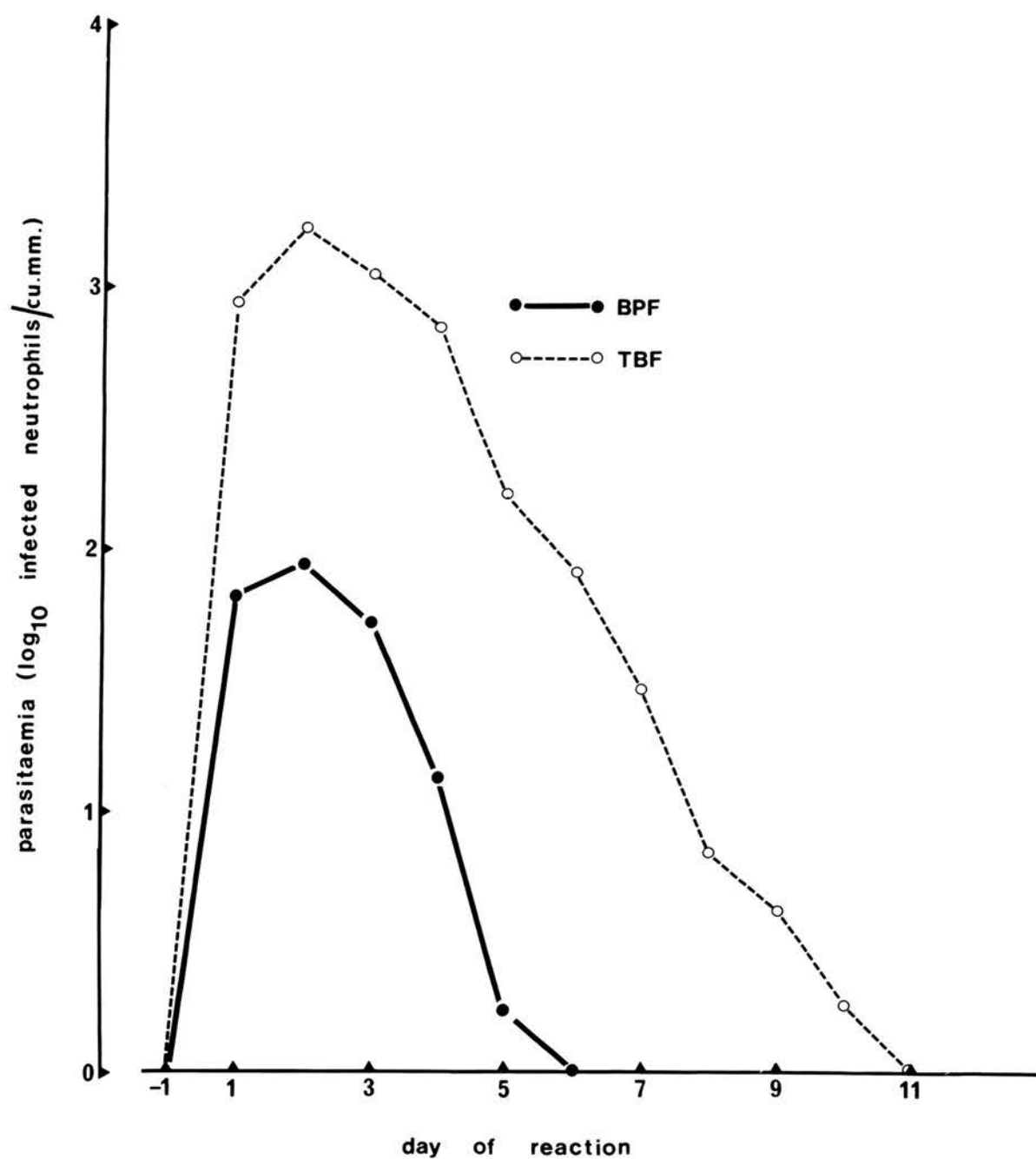


Fig. 29 MEAN PARASITAEMIC REACTIONS IN
SHEEP INFECTED WITH BPF AND TBF

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A COMPLEMENT FIXATION TEST FOR TICK-BORNE FEVER OF SHEEP

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SUMMARY

Antigens extracted from blood leucocytes of sheep infected with tick-borne fever fixed complement specifically in the presence of sera from sheep inoculated with infected allogeneic or autologous blood.

INTRODUCTION

Techniques for confirming a diagnosis of tick-borne fever are limited to the demonstration of the agent in blood granulocytes, either by staining (Foggie, 1951), or by direct immunofluorescence (Tuomi, 1967). This report extends the range of available techniques.

MATERIALS AND METHODS

Antigen. One strain of tick-borne fever was used, and infected sheep were bled at the peak of parasitaemia. The buffy coats were collected and suspended in a volume of Hank's balanced salt solution equal to approximately one hundredth of the original volume of blood. The cells were disintegrated by freezing and thawing or by sonication.

Sera. Sheep were inoculated intravenously with infected allogeneic blood collected in heparin and stored in dimethyl-sulphoxide at -76°C (Foggie, Lumsden & McNeillage, 1966). They were reinfected with stored infected allogeneic or autologous blood. Serum samples were collected at different intervals.

Complement fixation test. The technique described by the WHO Expert Committee on Respiratory Virus Diseases (1959) was followed. Antigens and sera were incubated with guineapig complement overnight. Then the sera were inactivated at 56°C for 30 minutes. Fixation, using 2–2.5 units of guineapig complement, was carried out overnight at 4°C . The haemolytic system consisted of sensitized 3 per cent sheep erythrocytes. The highest dilution of serum or antigen showing 50 per cent haemolysis after two hours was taken as the end-point.

RESULTS

Demonstration of antigen. Antigen extracted from blood leucocytes of 11 infected sheep reacted specifically with sera from sheep hyperimmunized with

infected autologous blood; no reactions occurred with preinoculation sera. Titres ranged from 1.9 to 2.8 \log_{10} units per ml. The percentage of infected granulocytes ranged from 15 to 60 and, in general, sheep with the higher parasitaemias gave higher yields of antigen. The correlation, however, was not significant ($F = 0.16$; d.f. 1, 7; $P > 0.05$). Antigens were not demonstrable in two sheep in which the percentage of infected granulocytes were 10 and 14 respectively.

Five batches of antigen were prepared by freezing and thawing the cells and eight by sonication. The titres were similar ($t = 1.749$; $P > 0.10$), the means and standard errors being respectively 2.16 ± 0.12 and 2.39 ± 0.11 \log_{10} units per ml.

Antigens prepared from the buffy coats of healthy sheep did not fix complement in the presence of hyperimmune serum.

Demonstration of antibody. Low dilutions of some preinoculation sera showed non-specific complement fixation but specific antibodies developed within 20 days and continued to rise (Fig. 1).

Seventeen sera samples were each tested against four different batches of allogeneic antigens and the coefficients of variation ranged from 0 to 10 per cent, the mean being 6 per cent.

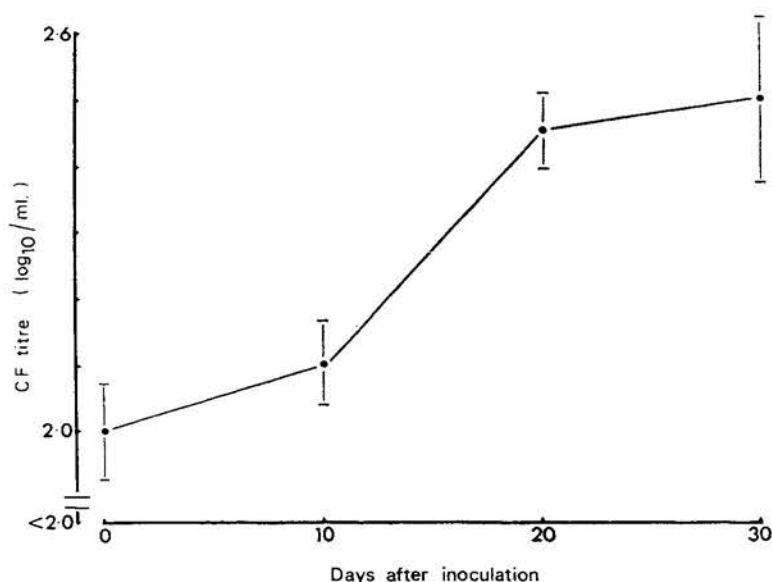


Fig. 1. Means and standard errors of complement fixing antibody titres of five sheep after allogeneic inoculation.

The specificity of the reactions was confirmed by testing, against allogeneic and autologous antigens, the sera of two sheep that were hyperimmunized by inoculation of their own infected blood that had been stored at -76°C . Differences between antibody titres to the two antigens were not significant ($t = 1.048$; $P > 0.05$) (Table I). Reinoculation, however, significantly raised the antibody titres ($t = 3.933$; $P < 0.01$), but further inoculations did not ($t = 0.275$; $P > 0.80$). The sheep showed no clinical signs on reinoculation.

TABLE I

TITRES OF COMPLEMENT FIXATION ANTIBODIES* IN THE SERA OF SHEEP GIVEN MULTIPLE INOCULATIONS OF THEIR OWN BLOOD INFECTED WITH THE TICK-BORNE FEVER AGENT

Serum origin	Antigen origin	Pre-inoculation serum	Allogeneic inoculation	Autologous inoculations		
				1	2	3
Sheep 16	Sheep 16	1.9	2.8	3.4	3.1	3.1
	Sheep 23	2.0	2.8	2.8	3.4	3.4
Sheep 23	Sheep 23	< 1.9	2.8	3.4	3.4	3.1
	Sheep 16	< 1.9	2.8	3.7	3.4	3.4

* Expressed as \log_{10} units/ml.

DISCUSSION

The tick-borne fever complement fixation test is specific and reliable. Antigens were recovered from sheep with parasitaemias greater than 15 per cent. The finding that sheep having complement fixing antibodies resisted challenge inoculations with no clinical signs suggests that the presence of complement fixing antibodies is related to immunity.

The pathogenesis and epidemiology of tick-borne fever are ill-understood and their study is hampered by the paucity of useful serological methods. The demonstration of specific complement fixation by tick-borne fever antigen and antibody therefore has some import in addition to extending the range of available diagnostic aids.

ACKNOWLEDGEMENT

This study was financed, in part, by a grant from the Overseas Development Administration, London.

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